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STUDIES ON AVIAN ENCEPHALOMYELITIS. I THE USE OF A SERUM-NEUTRALIZATION TEST IN THE DETECTION OF IMMUNITY LEVELS

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Received 23 Oct 1958

WORK in recent years has shown that an immunity is evoked following an outbreak of avian encephalomyelitis (AE).[2,6] Indeed, workers in California have instituted a large scale vaccination program in the breeding of stock of a commercial hatchery. By applying live virus to growing birds by wing-web inoculation, they obtained promising, though inconclusive, results.[6]

Evidence has been presented which indicates the development of serum antibodies following either a natural outbreak or an experimental infection with the AE virus.[1,2,4] Information is scarce on the degree of immunity necessary to protect aginst natural exposure.

Sumner and co-workers compared embryo susceptibility to egg-adapted AE virus using eggs from various sources.[8] It was apparent that the percentage of tested flocks producing susceptible eggs was low. It was not established whether the virus titers attained in the groups of eggs might be employed as criteria of the immunity levels in the parent stocks.

Contribution No. *1173* of the Department of Veterinary Science. Massachusetts Agricultural Experiment Station, Amherst.

A virus-neutralization test has been described for the detection of serum antibodies.[7] This test involves intraocular injection of chicken embryos. At best, this is a tedious and time consuming operation. A disturbing note mentioned by the authors was the abnormal number of "accidental survivals and deaths" encountered in their virus-serum titrations.

Wills and Moulthrop reported the propagation and egg adaption of the AE virus in chicken embryos inoculated via the yolk sac.[9] It is the purpose of this paper to describe the use of the yolk sac route for conducting serum-neutralization (SN) tests in embryonating eggs.

MATERIALS AND METHODS

Virus source. The AE virus employed in these studies was obtained from Dr. H. Van Roekel* as a brain suspension from the 150th intracerebral chick passage. This strain, which is highly virulent for young or adult birds, causes ataxia and death 7 to 21 days after intracerebral injection. A lethal dose-50 percent (LD_{50}) of 0.05 ml x $10^{-4.8}$ was observed following intracerebral injection of day-old chicks. Lesions typical of those described for AE[4] were found in brain sections of ataxic birds.

Experimental chickens and eggs. The first 12 embryo passages of the AE virus were carried out in eggs from a flock of Rhode Island Red (RIR) breeders maintained at the University of Massachusetts. Chicks for this experiment were hatched from the RIR flock and were held in isolation units. Subsequent passages, virus titrations, and SN tests were conducted in eggs from a privately owned flock of White Leghorns.

Egg adaption. Egg adaption of the AE virus was accomplished in the manner described by Wills and Moulthrop.[9] Six-day embryos were inoculated via the yolk sac with 0.2 ml of a 1:10 brain suspension (in nutrient broth). Brains were harvested 12 to 14 days postinoculation, ground with broth in a TenBroeck tissue grinder, and used as inoculum for the next passage. Harvesting was done using a 2 inch, 16 gauge needle

* Department of Veterinary Science, University of Massachusetts.

on a 10 ml syringe. The needle was inserted into the cranial cavity and the tip moved within the cranium while suction was applied with the syringe. In this manner approximately 0.7 to 0.8 ml of brain and cerebrospinal fluid was obtained from each embryo. All virus was stored at –20 C until used.

Virus titrations and serum-neutralization tests. Titrations were done using ten-fold serial dilutions of brain material in broth. Each dilution was injected into the yolk sacs of five 6-day embryos. Each egg received 0.1 ml.

SN tests were conducted as follows: Ten-fold serial dilutions of virus in broth were made starting with a 1 in 5 brain suspension. Plasma or serum (undiluted) and virus dilution were mixed at a 1:1 ratio giving final virus concentrations of 10^{-1}, 10^{-2}, etc. For virus controls, broth replaced the plasma. Virus-plasma mixtures were incubated at room temperature for 15 minutes, then injected into embryos in the manner described for virus titrations.

Embryos were examined live at 12 days postinoculation. Signs ranging from immobility, but with normal muscular development, to an almost complete absence of skeletal musculature were recognized as criteria of infection. These signs and the lesions encountered have been adequately described by Jungherr, *et al.*[3]

Embryo infective dose, 50 perecnt, (EID_{50}) titers were calculated according to the method of Reed and Muench.[5] Neutralization indices (NI) were calculated as the difference between the EID_{50} of the virus control group and the EID_{50} observed in a virus-plasma titration. The geometric mean titer was determined by averaging the neutralization indices of the birds in a group and was expressed with the standard deviation of the mean as GMT $\pm s_{\overline{x}}$

Intracerebral challenge of birds. Birds were challenged by intracerebral inoculation of a virus suspension to determine resistance to AE. Criteria of infection following inoculation consisted of ataxia or paralysis, absence of gross lesions on post mortem examination, and evidence of AE infection on histopathological examination of brain sections.

RESULTS

Egg adaption. In eggs from the RIR source, lesions typical of AE infection (see figure 1) were seen in the 2nd and all subsequent passages. The number of embryos with lesions never surpassed 77 percent in any passage. Eggs obtained from the White Leghorn flock, however, always gave 100 percent affected embryos when inoculated with harvests from the above passages. Moreover, EID_{50} titers ranged from 0.1 x $10^{-5.2}$ to $10^{-6.1}$. It was later found that the original brain suspension from the 150th chick passage gave an EID_{50} of $10^{-3.2}$ in the Leghorn eggs. It is doubtful than any "adaption" of this strain was necessary when "susceptible" eggs were used.

Growth curve following yolk sac inoculation. Because a high virus titer is advantageous for use in SN tests, two trials were conducted to determine the optimum time for harvesting from inoculated eggs. In each trial, 6-day-old embryos were inoculated in the yolk sacs with 13th and 15th passage material respectively. Each embryo received about 10,000 EID_{50}. At various intervals of incubation subsequent to inoculation, the brains from 6 embryos were harvested, pooled, and titrated in eggs. Results of these trials are found in table 1. Virus first appeared in the embryo brains between the 2nd and 4th days postinoculation as determined by the titrations of harvested material. Peak titers were reached after about 6 to 8 days followed by a gradual drop in titer through the 14th

TABLE I

AE virus titers in embryo brains subsequent to yolk sac inoculation of six day embryos

Days post Inoculation	*Inoculum 13th passage*	*15th passage*
2	0	0
4	1.8**	4.0
6	5.7	5.7
8	6.1	5.5
11	5.5	4.5
14	4.4	4.2

* Approx. 10,000 EID_{50} per embryo
** Log titers

Fig. 1. Eighteen-day embryos, skin removed. Embryo on right inoculated via the yolk sac on 6th day of incubation with 10,000 EID_{50} of 4th embryo passage AE virus.

day postinoculation. Because of these results, all virus subsequent to the 15th passage was harvested routinely on the 6th day postinoculation.

Titrations in embryos. Data is presented in table 2 to illustrate the consistency of virus titrations in embryos. Titrations were done at approximately weekly intervals from frozen aliquots of a 1 in 5 brain suspension of the 20th embryo passage.

Neutralization indices of normal plasma. Plasma was collected from 168 birds between 5 and 7 weeks of age. These birds had been hatched and raised in isolation and were later shown to be susceptible to AE infection. Neutralization indices ranged from –0.7 to+1.1. The GMT $\pm$ $s_{\bar{x}}$ was .14 $\pm$ .03 (figure 2).

TABLE II

Resuls of nine titrations of aliquots of AE virus
(brain suspension of 20th embryo passage)

Virus dilution	*Titration no.*								
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*
10^{-4}	7/7*	7/8	7/7	6/7	8/8	8/8	8/8	8/8	6/7
10^{-5}	7/8	6/8	8/8	4/8	4/7	4/8	7/8	5/8	7/8
10^{-6}	2/7	3/8	3/8	3/8	1/7	2/8	1/7	0/7	1/8
10^{-7}					0/8	0/8			
Log EID_{50}	5.6	5.5	5.8	5.2	5.2	5.2	5.5	5.2	5.4

* Number embryos affected/number examined.

Neutralization indices following experimental exposure to AE virus.

Trial 1. Two groups of ten 6-week-old RIR birds were employed in this trial. Group I birds were each injected intramuscularly (thigh) with 0.1 ml of a 1 in 5 suspension of uninfected brain material. Group II birds each received 0.1 ml intramuscularly of a 1 in 5 suspension of AE infected brain material (13th embryo passage). The dosage per bird was 50,000 EID_{50}. Serum-neutralization tests were conducted on blood samples collected just prior to inoculation and at 2 and 4 weeks postinoculation. At 4 weeks postinoculation the birds in both groups were challenged by intracerebral injection of 10,000 EID_{50} AE virus. Results of the serological studies are found in tables 3 (A) and 3 (B). Neutralization indices in the controls did not change significantly during the 4 week postinoculation period. Those of the AE inoculated group, however, rose to an average of 2.6. Within 3 weeks after challenge, all 10 of the control birds succumbed to AE infection. Of the 10 birds that had received AE virus intramuscularly, no signs were observed during a 4 week post challenge period.

Trial 2. Five 7-week-old birds were each inoculated in the wing-web (with a 2 pronged needle) with a suspension of AE infected brain material (approximately 6,000 EID_{50}). Five birds served as uninoculated controls. Serum-neutralization tests were done on samples collected preinoculation, 4 and 7 weeks postinoculation. Results are found in tables 4

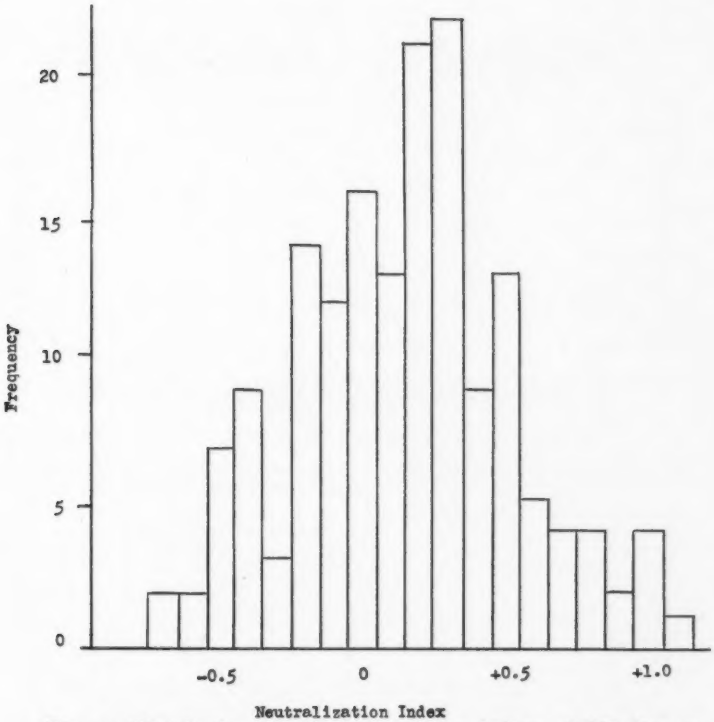

Fig. 2. Distribution of AE neutralization indices of 168 five to seven-week-old chickens susceptible to AE.

(A) and 4 (B). Although neutralization indices did not vary significantly in the control group, they rose to an average of 3.08 at 4 weeks postinoculation in the AE inoculated group. Three weeks later they had dropped but slightly to an average of 2.74.

DISCUSSION

The serum-neutralization test described herein offers a simple, uncomplicated method for measuring levels of immunity to avian encephalomyelitis. Virus titers produced by embryo-propagated material generally were found to exceed

TABLE III A

Neutralization indices following intramuscular injection of six-week-old chickens with 0.1 ml of uninfected brain suspension

Bird number	*Neutralization indices*		
	Preinoc.	*2 weeks*	*4 weeks*
1	0	.5	−.2
2	.2	.5	0
3	0	.1	.3
4	.3	.5	−.2
5	.6	.7	.3
6	.2		.2
7	−.6		−.7
8	.5		−.2
9	−.4		−.4
10	.8		.3
GMT $\pm s_{\bar{x}}$*	.16 ± .14	.24 ± .14	.08 ± .06

* Geometric mean titer ± standard deviation of the mean

TABLE III B

Neutralization indices following intramuscular injection of six-week-old chickens with 0.1 ml of AE infected brain suspension (10,000 EID_{50})

Bird number	*Neutralization indices*		
	Preinoc.	*2 weeks*	*4 weeks*
11	−.3	2.1	2.5
12	.4	3.1	2.8
13	−.6	2.6	2.5
14	−.1	1.8	2.2
15	.4	1.4	2.8
16	.3		2.6
17	0		2.6
18	.2		1.8
19	.1		3.4
20	−.2		2.8
GMT $\pm s_{\bar{x}}$*	−.02 ± .10	2.20 ± .30	2.60 ± .13

* Geometric mean titer ± standard deviation of the mean

an EID_{50} of 10^{-5}. A titer of this magnitude seems adequate for testing antibody levels of the order produced by either wing-web or intramuscular injection of live AE virus.

The importance of selection of egg source was well emphasized. Even low dilutions of the virus did not produce lesions in all of the eggs from one flock (RIR), whereas EID_{50} titers of over 10^{-5} were observed in eggs from a second flock (White Leghorn).

TABLE IV A

Neutralization indices following wing-web inoculation of seven-week-old chickens with 6,000 EID_{50} AE virus

Bird number	*Neutralization indices*		
	Preinoc.	*4 weeks*	*7 weeks*
1	.4	3.5	2.5
2	0	3.5	3.3
3	.1	2.0	2.1
4	1.0	3.4	3.0
5	.4	3.0	2.8
GMT $\pm s_{\bar{x}}$ *	.38 ± .39	3.08 ± .29	2.74 ± .31

* Geometric mean titer ± standard deviation of the mean.

TABLE IV B

Neutralization indices of seven-week-old uninoculated control chickens

Bird number	*Neutralization indices*		
	Preinoc.	*4 weeks*	*7 weeks*
6	–.4	.5	0
7	.2	.2	.5
8	.3	–.5	.2
9	.5	.2	.1
10	.2	.3	–.2
GMT $\pm s_{\bar{x}}$ *	.16 ± .5	.14 ± .17	.12 ± .12

* Geometric mean titer ± standard deviation of the mean.

Neutralization indices of 168 normal birds were found to range between –0.7 and +1.1. The average of +0.14 is close enough to 0 to suggest an absence of non-specific neutralizing substances in the plasma of these birds.

Following either wing-web or intramuscular injection of AE virus into susceptible birds, antibody titers rose consistently in all cases. The respone in the intramuscularly inoculated birds was evident after only 2 weeks. That these antibody titers were probably sufficient to ward off natural exposure is evidenced by the fact that the birds resisted the severe challenge of 10,000 EID_{50} injected intracerebrally.

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The advice and suggestions of Dr. M. Sevoian throughout the course of this work are gratefully appreciated. Thanks are due to Mrs. Judith Richardson for technical assistance.

SUMMARY

A serum-neutralization (SN) test for avian encephalomyelitis (AE) employing the yolk sac route of inoculation has been described. AE virus propagated in eggs injected by this route was found to titer between 10^{-5} and 10^{-6}.

Serum or plasma samples from 168 normal birds between 5 and 7 weeks of age had neutralization indices of -0.7 to $+1.1$ with an average of 0.14. Within 4 weeks after intramuscular or wing-web inoculation of suceptible birds, neutralization indices of 1.8 to 3.4 were detected with the SN test. Birds with neutralization indices of this order were capable of resisting intracerebral challenge with 10,000 EID_{50} of AE virus.

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EGG TRANSMISSION OF INFECTIOUS SINUSITIS FOLLOWING INOCULATION OF TURKEY BREEDERS IN EGG PRODUCTION*†

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OSBORN and Pomeroy[5] have recently reviewed the increasing amount of literature on infectious sinusitis of turkeys. Jerstad *et al.*[3] demonstrated egg transmission of infectious sinusitis in naturally infected turkeys but were unable to perpetuate the infection in the naturally infected stock beyond the third generation.

This paper reports egg transmission in breeders experimentally infected while in egg production.

MATERIALS AND METHODS

The basic plan of the prime experiment was 1) to rear and select two breeding flocks free of infectious sinusitis, 2) to rear two hatches from both flocks, 3) to inoculate one of the breeder flocks during egg production and to continue to hatch poults subsequent to infection, and 4) to rear each hatch from the two flocks separately to market age. This work covered one complete generation from day-old to the end of

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the breeder season and involved the second generation up to market age. Minimum market age was twenty weeks for females and about twenty-eight weeks for males unless otherwise stated.

Experimental subjects. Three lots of approximately equal number of Broad Breasted Bronze (BBB) turkeys, totaling 1229, were started at one day of age. They were progeny of three field flocks classified as free of infectious sinusitis on the basis of their history and a field inspection. They had been hatched at a commercial hatchery, either separately or with eggs from like flocks, and were reared away from other turkeys. No evidence of infectious sinusitis was observed in any of these turkeys. From this stock two breeder flocks of 120 females and 15 males each were selected. At selection time all breeders were tested and found negative to pullorum disease. At the same time they were vaccinated for erysipelas, pox, and Newcastle disease. Lighting, feeding, and management of breeders were comparable with commercial practices in this area.

Inoculation of breeders. A field strain of an infectious sinusitis agent propagated in turkey sinuses was used. Exudate was aspirated from infected sinuses periodically, cultured, and stored in a freezer. The frozen exudates that had shown no bacterial growth on tryptose-enriched nutrient agar slants were thawed, pooled, and diluted 1:100 in physiological saline. The females of one flock, then 41 weeks old, were injected with 1.0 ml of the diluted sinus exudate into one posterior thoracic air sac and with 1.0 ml into each sinus. The other flock served as a noninfected control.

Hatching, brooding, and rearing. Two hatches were set from each flock prior to inoculation. Beginning 17 days after inoculation, all eggs suitable for hatching were saved and set in six more hatches as shown in table 1. Progeny of the control flock were hatched, brooded, and reared separately from progeny of the infected flock, except that in the last two hatches one-half of the eggs from the noninfected flock were hatched with the eggs from the inoculated flock. The poults were brooded separately from each other and the remaining poults from the control flock.

All poults hatched were observed for clinical evidence of the disease. Inoculation of material from infected poults into the sinuses of test poults was used to confirm the diagnosis.

Sulfaquinoxaline was administered in the water as required to control brooderhouse coccidiosis. Histomonastatic drugs were sporadically used on the range to control infectious enterohepatitis. No other drugs were used.

Confirming experiments. To confirm observations in the prime experiment, two BBB flocks of 10 females in egg production and two males each were set up in succeeding years at different seasons. These birds were about 9 weeks older when inoculated and had been in production longer than those in the prime experiment. A lower respiratory infection typical of infectious sinusitis was induced by intratracheal inoculation of isolates of pleuropneumonia-like organisms (PPLO) from chickens. Each flock was inoculated with a separate isolate, one more pathogenic than the other. Separate weekly hatching and brooding of their progeny followed for 174 days after inoculation of the breeder females of one flock and 221 days after breeder inoculation of the other. The same basic observations were made as in the prime experiment above. In addition, all poults that died in the brooder were cultured for PPLO and all poults were tested by serum plate PPLO agglutination test of Adler[1]. Isolation of PPLO or positive serum plate agglutination reactions were considered as positive evidence of egg transmission.

RESULTS

Three of six hatches produced from the infected flock of the prime experiment after inoculation showed evidence of infectious sinusitis at seven or eight weeks of age as shown in table 1. The other three hatches showed no evidence of infectious sinusitis up to market age. In two of the infected hatches, the disease was not a problem because no spread occurred. A brooderhouse outbreak of infectious sinusitis occurred in the hatch from eggs laid 73 to 93 days after breeder inoculation. The poults from the control flock, hatched with poults from the infected flock but brooded separately, showed no evidence of infectious sinusitis.

TABLE I

Incidence* of infectious sinusitis in the prime experiment in hatches subsequent to experimental inoculation of female breeders with an agent in the form diluted sinus exudate

Hatch No.	Days after inoc. eggs were laid	Age of poults when symptoms were noted	No. initially infected/no. started (%)	Remarks
4	17 to 30	8 weeks	1/93 (1.0)	Affected poult had swollen sinus; no spread.
6	31 to 44	7 weeks	2/93 (2.0)	Two "coughers" which died of infectious enterohepatitis; no spread.
7	45 to 58	No symptoms	0/95 (0.0)	Juxtaposition on range with only a fence between since hatch 8 was 11 weeks old.
8	59 to 72	No symptoms	0/68 (0.0)	
9	73 to 93	7 weeks	10**/81 (12.4)	Developed into a flock infection.
10	94 to 100	No symptoms	0/61 (0.0)	
TOTAL			13/491 (2.65)	

*To market age; youngest females were marketed at 20 weeks of age, some held to 24 week of age; males marketed at about 28 weeks of age.

**Estimated number.

All cases of infectious sinusitis in the experimental hatches were confirmed by sinus injection of test poults, except hatch 6, because it was desired to preserve the focus of infection to allow natural spread. The subsequent death of the only two symptomatic individuals and failure of the disease to spread precluded planned confirmation.

In one of the flocks in the confirming experiments inoculated with the more pathogenic of two cultures, three inoculated female breeders died from the disease. As shown in table 2, poults were hatched weekly from eggs laid 1 to 10 and from 95 to 221 days after breeder inoculation. Because of the severity of the disease induced by breeder inoculation, only one poult was hatched from this flock from eggs laid from 11 to 94 days after inoculation. In the other flock, which was inoculated with a less pathogenic culture, poults were hatched regularly from eggs laid 1 to 174 days after breeder inocu-

TABLE II

Incidence of infectious sinusitis in hatches (to 14-17 weeks of age) subsequent to experimental inoculation of female breeders in the confirming experiment which received the most pathogenic PPLO culture

Weekly hatch no.*	Days after inoc. eggs were laid	No. of poults	Serum plate PPLO test @ 3 to 6 and 14 to 18 wks.	PPLO isolations from dead poults
37 and 47	1 to 10	8	Negative	Negative
57 to 167	11 to 94	1	No test, poult died	Negative
177	95 to 101	6**	Positive	3 dead first week; all pos.
187 to 217	102 to 129	11	Positive†	Negative
227 to 357	130 to 221	98	Negative	Negative

* Hatches are numbered consecutively by weeks from the first Monday of the year. The first two digits indicate week; last digit indicates 1957.

**Symptoms of lower respiratory form of infectious sinusitis noted at hatching time and in the brooder in 5 of these 6 poults.

†Only one poult was hatched in the first 3 of this series of 4 weekly hatches. Two of these showed suspicious reactions at 3 and 4 weeks of age. At 16 weeks a positive reaction was obtained from one of them. At 14 weeks of age, the fourth hatch which was negative at 3 weeks of age was positive. Because hatches of this series were combined prior to 8 weeks of age, cross infection may have occurred.

lation (table 3). Progeny of these flocks hatched from eggs laid 91 to 97 and 95 to 101 days after inoculation of the breeders were the first involved in egg transmission. Evidence of egg transmission was based on PPLO isolations, clinical symptoms, and the results of the PPLO serum plate agglutination test, alone or in combination. After the initial egg transmission at 91 to 101 days after breeder inoculation, infected progeny were hatched from eggs laid up to 132 days after breeder inoculation, but hatches free of evidence of the disease were interspersed between infected hatches.

All poults from the control flock remained free of the disease up to market age, including two hatches of noninfected poults hatched with eggs from the inoculated flock.

DISCUSSION

Because Mataney *et al.*[4] reported a high incidence of egg transmission of infectious sinusitis, more egg transmission

TABLE III

Incidence of infectious sinusitis in hatches (to 14-18 weeks of age) subsequent to experimental inoculation of female breeders in the confirming experiment which received the least pathogenic PPLO culture

Weekly hatch no.*	Days after inoc. eggs were laid	No. of poults	Serum plate PPLO test @ 3 to 6 and 14 to 18 weeks	PPLO isolations from dead poults
147 to 267	1 to 90	120	Negative	Negative
277	91 to 97	16	Positive	Negative
287	98 to 104	12	Negative at 3 weeks; Pos. at 15 wks.	Positive
297	105 to 111	7	Positive	Negative
307 & 317	112 to 125	19	Negative	Negative
327	126 to 132	6	Negative	Positive
337 to 387	133 to 174	25	Negative	Negative

* Hatches are numbered consecutively by weeks from the first Monday of the year. The first two digits indicate week; last digit indicates 1957.

than occurred in these experiments might be anticipated. However, basic differences in experimental design should be considered. First, there is a possible difference between a field strain, sinus propagated or a PPLO culture and an agent grown on egg embryos. Secondly, in this experiment, observation of progeny began after hatching, whereas the above writers made observations up to hatching time only.

The lack of spread of the disease in hatches in the prime experiment in which the disease was manifested by swollen sinuses is not surprising; this is commonly observed. However, spread of the disease is more apt to occur when coughing is observed, but the disease did not spread in hatch 6. Despite these results, the presence of the infection in a hatch must be considered as a potential source of a possible outbreak.

The most striking observation in these experiments was that little or no consistent egg transmission with brooderhouse outbreaks of the disease was observed other than in hatches from eggs laid months after breeder inoculation. The periods after breeder inoculation defined herein were dependent on the schedule of collection and holding of eggs before setting

and the relationship of the day of inoculation to day of setting. In the prime experiment, eggs were held for 7 or 14 days before setting except that eggs laid 73 to 93 days after breeder inoculation were held 21 days. Weekly hatches in the confirming experiments were designed to give more precise definition over a longer period, but such few poults were obtained in many hatches that the chance of demonstrating egg transmission did not appear likely. That it was demonstrated in poults from both flocks is, therefore, considered significant.

Table 2 shows that egg transmission occurred in a hatch from eggs laid 95 to 101 days after breeder inoculation. This hatch showed clinical symptoms of the disease, the agglutination test was positive, and PPLO were isolated. In table 3 the serum plate PPLO agglutination test indicates egg transmission from eggs laid 91 to 97 days after breeder inoculation in the second confirming experiment. Further evidence of egg transmission occurs in tables 2 and 3 up to 132 days after breeder inoculation. Not all hatches in the series up to 132 days were involved. Termination of the prime experiment 100 days after breeder inoculation precludes comparison beyond that time (table 1). However, the hatch from eggs laid 94 to 100 days after breeder inoculation did not show evidence of egg transmission although it occurred in the immediate preceding hatch from eggs laid 73 to 93 days after breeder inoculation. This pattern of transmission in one hatch with no transmission in adjacent weekly hatches appears in all inoculated flocks. Symptomatic evidence of the disease occurred in the egg transmission from eggs laid 73 to 93 days after breeder inoculation in the prime experiment listed in table 1 and from eggs laid 95 to 101 days after breeder inoculation in the confirming experiment listed in table 2. Symptoms were absent in the egg transmission from eggs laid 91 to 97 days after inoculation of the breeders in table 3.

It is noteworthy that in all three flocks, eggs laid from 91 to 101 days after breeder inoculation were included in hatches involved in egg transmission. Since the three experimental breeder flocks were inoculated in different years, at different seasons with separate agents of varying pathogenicity by two

routes, a relationship between time of breeder inoculation and a period of consistent egg transmission is evident. Thus, it appears that a partial pattern of egg transmission has been revealed in these experiments. Eggs laid about 90 days after inoculation of breeders in egg production are likely to be involved in egg transmission resulting in brooderhouse outbreaks. Realization of this possibility considerably narrows the efforts required to achieve planned experimental production of brooderhouse outbreaks of the disease, and helps explain field observations and literature reports which appear contradictory.

Field observations of infectious sinusitis and interviews with experienced hatcherymen have not revealed consistent proof that infectious sinusitis is egg transmitted in face of the many instances of free hatches from known infected flocks. The overall results of these experiments plausibly explain field observations since both free and infected progeny were produced by infected breeders. Furthermore, infected progeny did not always precipitate a brooderhouse outbreak of the disease. The results also suggest a partial explanation for the difficulty encountered by Grumbles and Boney[2] in demonstrating egg transmission experimentally and for the inability of Jerstad *et al.*[3] to perpetuate the disease in naturally infected stock.

SUMMARY AND CONCLUSIONS

Females in one of two flocks of turkey breeders in egg production and with no history or evidence of infectious sinusitis were inoculated intrasinusoidally and into one posterior thoracic air sac with a field strain of the infectious sinusitis agent in the form of diluted sinus exudate. Six hatches from the two flocks were made from 17 to 100 days postinoculation. Two hatches were made from both flocks prior to inoclulation of one of them. In two confirming experiments with flocks of 10 females and 2 males each, weekly hatches followed intratracheal infection of the females with PPLO cultures for 174 days in one flock, for 221 days in the other.

One-half or more of the hatches from all infected flocks

produced poults free of clinical evidence of the disease. In some hatches, egg transmission represented potential, rather than actual significance because clinical evidence of the disease was limited to individual poults and did not spread to penmates. The limited number of hatches in which a clinical outbreak of the disease occurred in the brooderhouse appeared to bear a relationship to the time of inoculation of the female breeders, since in each of these cases, eggs laid in the neighborhood of 90 days after breeder inoculation were included in the setting.

Poults hatched from uninoculated control flocks showed no evidence of infectious sinusitis.

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THE CLINICAL COURSE OF INFECTIOUS SINUSITIS IN EXPERIMENTALLY INFECTED TURKEYS

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THE clinical course and the effect of infectious sinusitis in individual turkeys or flocks deserves further delineation. Hinshaw's[4] brief discussion on the course and mortality is generally accepted: "Sinusitis of the obviously contagious type runs a chronic course and may exist in a flock for weeks. Although the number of deaths may be less than in some more acute diseases, the financial loss may be greater. Failure to gain weight accounts for as much damage as does mortality."

Boyer *et al.*[2] in one of two experiments, studied the course of infectious sinusitis of turkeys over a 3 week period and observed recovery from the lower respiratory form of the disease, with practically no recovery from swollen sinuses. In an experimental inoculation of the infectious sinusitis agent into the posterior thoracic air sac, the same writers produced respiratory symptoms in seven days. The incubation period of the disease was found by Jerstad *et al.*[6] to vary considerably and may be dependent on agent virulence, age of

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bird when infected and the route by which the agent gains access. Hitchner[5] also suggests that susceptibility of birds may increase with age, but that his results may have been due to increased agent virulence.

Infectious sinusitis, a term suggested for this disease by Dickinson and Hinshaw[3] to differentiate it from vitamin A deficiency and fowl coryza, may show two distinct clinical manifestations or a combination of the two in individual turkeys or flocks. Distended infraorbital sinuses which result from the collection of a characteristic semigelatinous exudate are seen in the relatively noncontagious sinus form of the disease. The contagious lower respiratory form of the disease is manifested by a persistent characteristic moist cough which may be aggravated to the point of suffocation with increased use of the respiratory organs. Misunderstanding of observations may result unless reported findings are related to one or the other or both of these separate clinical manifestations of the one disease.

Observations to be reported in this paper deal with the clinical course and effect of the disease and includes limited serological studies.

MATERIALS AND METHODS

A breeder flock of Broad Breasted Bronze turkeys in full egg production was inoculated with an infectious sinusitis agent in the form of a 1:100 dilution of sinus exudate into one posterior thoracic air sac and both sinuses. Only the females, numbering about 100, were inoculated. An uninfected flock of like size was maintained as a control. Performance of the breeders was measured by egg production and hatch records. The course of the disease was followed clinically.

Advantage was taken of an outbreak of infectious sinusitis in one hatch from the inoculated breeders to study an egg transmitted infection in poults. Concurrently, an attempt was made to separate out asymptomatic individuals. The study was initiated when the poults were 12 weeks old, five weeks after symptoms were noted. Asymptomatic poults were moved to a separate colony house. Poults that coughed or

had a sinus swelling remained in the colony house in which all had been started. When symptoms of infectious sinusitis were observed in poults selected as asymptomatic, the affected individuals were removed. The progress of the disease in the two houses was followed until the poults were 24 weeks of age.

Serological tests. Beginning at 12 weeks of age, and periodically thereafter to 20 weeks of age, the infected poult flock was tested by the serum plate agglutination test (PPLO test) of Adler[1] modified to the extent that readings were made up to eight minutes after mixing of serum and antigen. The PPLO antigen was supplied by Dr. H. E. Adler of the University of California. One house of poults from the non-infected flock hatched with the poults that had the infectious sinusitis outbreak and brooded separately was tested to check for possible inapparent infection.

Serum samples were also tested from females in the inoculated breeder pen. Number of samples and times at which they were taken appear in table 5.

RESULTS

Effect of inoculation on breeders. Coughing and sinus swellings were noted in the experimental females six days after inoculation with the infectious sinusitis agent. Coughing characteristic of infectious sinusitis was noted in the non-inoculated males on the 26th day after inoculation of the females, but may have occurred earlier. None of the males showed any sinus swellings. Six weeks after inoculation, coughing had almost completely subsided in all birds.

As the sinuses became so greatly distended that sight was impaired, treatment with four percent silver nitrate solution in the manner of Dickinson and Hinshaw[3] became necessary. Groups of birds were treated on the 26th, 33rd, and 46th days postinoculation. Only one sinus of each bird was treated at one time to avoid temporary blindness in both eyes that may result from the action of the silver nitrate treatment. The other sinus was treated one to two weeks later. The swollen sinuses were satisfactorily controlled as a result of these treatments.

TABLE I

Course of infectious sinusitis in poults from 12 to 24 weeks of age

Symptoms*	Ages examined, number and percent showing symptoms									
	12 wks.		16 wks.		18 wks.		20 wks.		24 wks.	
	No.	%	No.	%	No.	%	No.	%	No.	%
Sinus swelling	17	43.6	22	56.4	22	56.4	22	56.4	21	53.8
Cough	17	43.6	2	5.1	1	2.6	0	0.0	0	0.0
Sinus swelling and a cough**	5	12.8	0	0.0	0	0.0	0	0.0	0	0.0
No symptoms	0	0.0	15	38.5	16	40.8	17	43.6	18	46.1
TOTAL	39		39		39		39		39	

*Symptoms first observed at 7 weeks of age.
**Sinus swellings persisted and are included in the "Sinus swelling" group at 16 weeks of age; cough did not persist.

TABLE II

Course of infectious sinusitis in asymptomatic poults selected from an infected group at 12 weeks of age

Symptoms*	Ages examined, number and percent showing symptoms									
	12 wks.		12½ wks.		13½ wks.		15 wks.		16, 18, 20 & 24 weeks	
	No.	%	No.	%	No.	%	No.	%	No.	%
Sinus swelling	0	0.0	1**	2.6	0	0.0	0	0.0	0	0.0
Cough	0	0.0	6	15.8	1	3.2	1	3.3	0	0.0
Sinus swelling and a cough	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
No symptoms	38	100.0	31	81.5	30	96.7	29	96.6	29	100.0
TOTAL	38		38		31		30		29	

*Symptoms first observed in group from which selected at 7 weeks.
**Birds were removed from house when symptoms were noted.

Egg production in the inoculated flock was consistently 10 to 20 percent under that of the control flock except in the next to final week of the experiment. The decline does not take into account the fact that the drop in production following inoculation was initially accompanied by an increase in white and soft-shelled eggs unsuitable for hatching. Hatchability in biweekly hatches dropped drastically following inoculation to around 20 percent and reached a low of about 16 percent. Meanwhile, hatchability in the control flock reached a high of 60 percent and was consistently above 50 percent in all biweekly hatches.

Course of the disease in poults. The progress of infectious sinusitis in the poults following separation of asymptomatic from symptomatic poults is shown in tables 1 and 2. Sinus swellings persisted throughout the experiment in all but 1 of 22 birds. Of the original 17 poults which had lower respiratory symptoms and of the 5 with these symptoms plus swollen sinuses at 12 weeks of age, only 1 had a noticeable cough at 18 weeks of age. This turkey had no symptoms when examined at 20 weeks of age. Thus, coughing in individual birds was of limited duration as was the case in the inoculated breeders.

In the asymptomatic group, 8 poults exhibited a mild cough and 1 developed a swollen sinus between 12½ and 15 weeks of age. After these were removed, the other turkeys remained free of both sinus swellings and coughing to 24 weeks of age.

Serological tests. The results of periodic PPLO tests of poults are given in tables 3 and 4. Although the disease was first recognized at seven weeks of age, complete agglutination did not occur until 16 weeks of age. In individual poults, the reactions to the tests were not consistent from one testing period to another, nor was there perfect correlation with symptoms.

PPLO tests of poults from noninfected breeders, hatched with infected poults and separately brooded, were negative to 24 weeks of age and these birds showed no symptoms of infectious sinusitis.

TABLE III

Serum plate agglutination tests for PPLO of poults showing symptoms of infectious sinusitis at 12 weeks of age

Symptoms at 12 weeks	Percent negative (N), incomplete (I), or positive (P) at stated ages											
	12 weeks			16 weeks			18 weeks			20 weeks		
	N	I	P	N	I	P	N	I	P	N	I	P
Sinus swelling	70.6	29.4	0.0	23.5	29.4	47.0	35.2	35.2	29.4	29.4	29.4	41.2
Cough	35.2	64.8	0.0	23.5	47.0	29.4	17.6	64.8	17.6	0.0	47.0	53.0
Sinus swelling and cough	40.0	60.0	0.0	20.0	20.0	60.0	20.0	40.0	40.0	40.0	20.0	40.0
TOTAL	51.2	48.8	0.0	23.0	36.0	41.0	25.6	48.8	25.6	17.9	36.0	46.1

TABLE IV

PPLO agglutination test results in asymptomatic poults selected from an infected group at 12 weeks of age

Percent reaction at stated ages*												
12 weeks			16 weeks			18 weeks			20 weeks			
N	I	P	N	I	P	N	I	P	N	I	P	
65.5	34.5	0.0	34.4	31.1	34.5	24.1	34.5	41.4	20.6	44.8	34.5	

*Poults which developed symptoms and were removed from the house are not included in these tests.
N = No agglutination I = Partial agglutination P = Complete agglutination

Results of PPLO tests of breeders at various intervals are in table 5. Complete agglutination developed by 46 days after inoculation. Incomplete agglutination was observed in samples taken 26 and 33 days after inoculation.

TABLE V

PPLO agglutination tests of breeders inoculated with infectious sinusitis agent at 41 weeks of age

No. tested	Days before (—) or after (+) inoculation	Percent Negative	Incomplete	Positive
10	—28	100.0	0.0	0.0
42	+26	78.5	21.3	0.0
42	+33	90.4	9.5	0.0
21	+46	14.7	28.0	57.1
8	+102	75.0	0.0	25.0

DISCUSSION

Course of disease. The inoculation of the experimental females produced an acute infection which spread readily to the uninoculated males. Since swollen sinuses were controlled by treatment, the disease became essentially a lower respiratory infection. Its course, as a flock infection, was practically over in six weeks. This is a much shorter period than is often observed in field flock infections and undoubtedly resulted from the simultaneous infection of all the females. Coughing in individual poults was also of limited duration, but swollen sinuses generally persisted to market age. These findings confirm and extend those of Boyer *et al.*[2] and correspond to observations of the writers in other experiments involving inoculation of either exudates from active cases, agents grown on egg embryos, or cultures of pathogenic PPLO.

The incubation periods observed correspond in a general way to those of Jerstad *et al.*[6] and Hitchner[5], but they do not determine the influence of agent virulence, route of inoculation or possible difference in susceptibility owing to the age of the turkey. Relapses were not observed in this experiment.

Serology. PPLO test reactions in both the breeders and poults were preceded by characteristic symptoms in this experiment. On the basis of PPLO tests, the attempt to separate out asymptomatic birds at 12 weeks of age demonstrated no practical advantage, since by 20 weeks of age, there was

no essential difference in test reactions in the two groups. Such an effort may have had a better chance of success if the attempt had been made as soon as the infection was noted.

The similarity in the test results in the poults listed in tables 3 and 4 suggests that poults selected as asymptomatic at 12 weeks of age may have shown undetected symptoms prior to that time and had recovered. Observations in an unreported experiment give some credence to this possibility. Otherwise, the possibility of inapparent infections in individuals during an acute outbreak must be considered.

SUMMARY AND CONCLUSIONS

Infectious sinusitis was induced in turkey breeder females in full egg production by inoculation of diluted sinus exudate of an agent from a field case into an air sac and the sinuses. Swollen sinuses and coughing resulted in six days. Only the lower respiratory form of the disease evidenced by coughing spread to the uninoculated males in the flock. Swollen sinuses were controlled by silver nitrate treatment. Six weeks after inoculation, coughing was no longer in evidence. Egg production and hatchability percentages were drastically reduced for an extended period as a result of the inoculation.

In infected progeny, coughing was observed in the flock for a longer period than in the inoculated breeders. However, individual poults showed about the same maximum period of coughing while untreated swollen sinuses persisted.

On a flock basis, complete reactions to the serum plate agglutination test for pleuropneumonia-like organisms were preceded by characteristic symptoms in breeders and poults.

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CAGE LAYER FATIGUE (CAGE PARALYSIS)

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CAGE layer fatigue, frequently called cage layer paralysis, manifested itself as a significant disease problem soon after the practice of keeping layers in individual cages became popular in the south and southwest in 1954-55. From discussions with veterinarians, who have many years of experience in the field of poultry diseases, it appears that the same, or a similar condition, appeared many years ago with the first usage of the laying batteries in the thirties. No doubt, cage layer fatigue is not a new condition, rather it is one that has become important because of the extensive use of a different system of housing and management.

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There is a paucity of published information on this condition. It is not included in Diseases of Poultry by Biester and Schwarte[2] and is only briefly mentioned in Diseases and Parasites of Poultry by Barger, Card and Pomeroy[1]. Grumbles[7] recognized cage layer fatigue as a significant disease problem in Texas in 1955. Couch[3] has described the condition as some type of leg trouble that disappears when the affected birds are removed from cages. Couch[4] also stated that bone ash, blood calcium and blood phosphorus values on selected early cases were all within the normal range, indicating that the affected birds did not have rickets. Couch[4] has associated the condition known as "fatty liver disease" with cage paralysis. However, there is no evidence that the two conditions are related. Fisher[5] (personal communication) stated that in his experience, cage fatigue has not occurred when the calcium level of the diet was maintained at 2.5 percent. He further stated that the condition is probably not restricted to caged birds, but that it cannot be clearly diagnosed in birds maintained on floors.

Hartman and King[8] stated that most of the cage replacements in the southern states are grown on litter and on range while in California they are usually grown entirely on wire. They stated that in California, cage layer fatigue is not known and suggested that range and litter raised pullets may have difficulty in adjusting to wire floors.

Francis[6] reported the condition in caged birds in New Mexico and accumulated data which indicate a difference in the occurrence of the condition among strains of birds. He also reported that recovery of affected birds depends upon the strain of birds and the early removal from the cages.

Incidence and nature of the disease. The number of cases of cage layer fatigue recognized by the diagnostic laboratories in Texas for the past four years is as follows:

1954-55	15
1955-56	12
1956-57	12
1957-58	19

These figures are only an indication of the true incidence of the condition because, in many instances, the owner or a field service man is able to recognize the disease and specimens are not submitted to a diagnostic laboratory. Also, many flock owners have come to expect a certain amount of cage paralysis and ignore the condition in individual birds.

Cage layer fatigue occurs more often in young pullets that are producing at a high rate and on a good feed efficiency ratio. The disease apparently occurs more frequently in birds that are producing at a high rate during the summer months. Those individual hens that become affected are almost always the better layers. Many birds will lay on the same day that they develop paralysis and some will continue to lay for a day or two after becoming paralyzed. Poor shell quality has not been associated with the condition.

The symptoms are simply those of a paralysis. The birds are found down in the cage and are unable to stand. Affected birds appear alert and will continue to eat and drink if food and water are within easy access. The incidence in the flock is from less than 1 percent to as high as 20 percent in rare instances. From 80 to 90 percent of the affected birds will recover within 4 to 7 days if they are removed from the cage early and placed on the floor or in an outside pen. Some cage layer operators simply place a piece of cardboard or metal in the cage to provide a solid floor.

Necropsy lesions. Many affected birds when presented for examination have one or more broken bones in the wing or leg. Bones are often broken in the process of destroying a bird for examination.

The primary characteristic of cage fatigue is the fragility of the bones. The walls of the bones are extremely thin, but they are not soft or rubbery as they often are in rickets. In contrast, they are hard, brittle and are easily broken. In some birds, the long bones in the wings or legs can be crushed by simply applying pressure with the thumb and index finger. In many birds, the ribs are deformed and often appear to have been broken.

Birds that have been affected for several days may be-

come emaciated. Usually there are no specific lesions in the visceral organs.

Diagnosis. Diagnosis in an individual bird may be difficult. However, when the condition occurs as a flock problem, the history, symptoms and lesions are usually sufficient to allow an accurate diagnosis. Cage fatigue must be differentiated from neural lymphomatosis. The absence of lesions in the nerves and the fact that most affected birds removed from cages recover within a few days, usually eliminates leucosis from consideration.

SUMMARY

The incidence of a condition known as cage fatigue or cage paralysis has increased with the rapid increase in the practice of keeping layers in individual cages. The disease has become an important problem in cage layer operations in the south and southwest.

The exact cause of the factors that influence its occurrence are not known. It is apparently associated with some nutritional factor or factors and is evidently influenced by metabolic and physiological functions.

The condition can usually be diagnosed by the flock history, symptoms, gross lesions and the elimination of other common causes of paralysis.

Most affected birds recover if they are removed from the cage in the early stages of the disease and placed on the ground or floor. Specific recommendations for prevention must await additional information about the causative factors.

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IMMUNITY STUDIES ON AVIAN INFECTIOUS SYNOVITIS

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AVIAN infectious synovitis was first described by Wills[9,10] and Olson[3,9] in 1954. Since that time the disease has been studied by numerous investigators and several descriptions of the disease and of the causative agent have been published.[1,2,5,7,8] However, the majority of the publications have dealt with therapy studies. The serologic relationships between infectious synovitis and fowl pox, Newcastle disease, chronic respiratory disease and infectious bronchitis have been briefly studied by several investigators.[5,6,1,7] Reports on the immunity produced in chickens by an attack of the disease could not be found in the available literature.

MATERIALS AND METHODS

The Bridges strain of infectious synovitis agent isolated by Wills in 1954[9] from a flock of infected broilers was used in all of the experiments with one exception. Strain 105 obtained from Dr. M. S. Cover of the University of Delaware was used in one study. The Bridges strain consistently produced a high mortality in chickens when inoculated by the intramuscular or intravenous route. Strain 105 produced

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a variable mortality and in many instances only a transient lameness in chickens when inoculated by the intravenous or intramuscular route. Material used in making vaccine or for challenge purposes consisted of liver and spleen material collected from birds dead or moribund of the disease, or yolk material collected from chicken embryos which had died as a result of infection with the etiological agent of avian infectious synovitis. The material was checked for bacterial contamination by the inoculation of tryptose agar slants and dextrose phenol red broth enriched with two percent (Difco) PPLO serum fraction. Six-day-old chicken embryos were used to propagate the agent.

This study was divided in two parts. The first was a study of the natural immunity produced by the disease. The second was designed to determine if immunity might be stimulated by formalinized tissue or egg propagated vaccines.

Natural immunity. Natural imunity to the disease was studied in three groups of chickens. In these studies, the birds were infected by an injection of liver or spleen taken from birds dying of infectious synovitis or yolk material from embryos killed by the infectious synovitis agent. The birds surviving this exposure were later combined with an equal number of controls and the entire group challenged with infectious yolk or chicken tissues.

The first natural immunity study was carried out with 100, thirteen-week-old hybrid male chickens which were wing banded and divided into two groups. The initially infected group was placed in colony type brooder houses while the control group was maintained in battery brooders. Both groups were fed a commerical laying ration without antibiotics. Initial infection was accomplished by the intramuscular injection of 0.5 ml of a 50 percent suspension of liver and spleen in nutrient broth. The tissues were from birds dying of infection with the eighth consecutive bird passage of the Bridges strain. Following the appearance of symptoms and mortality, soluble tetracycline was administered in the drinking water at the rate of 400 mg per gallon and the more severely affected birds were injected with 25 mg of oxytetracycline in oil.

The second natural immunity study was a replication of the first. One hundred, thirteen-week-old, hybrid male chickens were wing banded and divided into two groups. The birds that were initially infected were placed in colony type brooder houses while the control group was maintained in battery brooders. Both groups were fed a commercial laying ration which did not contain antibiotics. The initial infection was produced by the intramuscular injection of 0.5 ml of a 50 percent suspension of liver and spleen in nutrient broth. The agent used was the ninth consecutive bird passage of the Bridges strain. The group was visibly affected and five birds were found dead on the eighth day following infection. To avoid excessive mortality, treatment was started consisting of one gram of tetracycline in each gallon of drinking water and an injection of 25 mg of oxytetracycline in oil for each of the more severely affected birds. Six weeks after the initial infections the survivors were combined with twenty-six control birds of similar age, breeding and nutrition. The entire group was then infected by an intramuscular injection of 0.5 ml of a 50 percent suspension of hock exudate in nutrient broth which had been stored at –10 C for 21 days. The exudate was from birds dying of infection with the twelfth consecutive bird passage of the Wills strain. The birds were placed in the colony type brooder house in which the previously infected birds had been housed.

The purpose of the third natural immunity study was to investigate the possibility that a strain of infectious synovitis might become attenuated in its virulence for chickens by repeated passage in embryonating chicken eggs. Fifty, thirteen-week-old hybrid male chicks were wing banded and divided into two groups which were reared and maintained under conditions similar to those described in the two preceding studies. Initial infection was accomplished by the intramuscular inoculation of one ml of yolk material harvested from chicken embryos which had died from infection with the seventh to ninth embryo passages of strain 105. Nine days following the injection, three birds manifested a transient lameness which had disappeared by the twelfth day post inoculation. The

previously injected and control birds were combined and challenged on the 43rd day following the initial injection by the intramuscular injection of one ml of a physiological saline solution suspension of liver and spleen from birds dying of infection with the twelfth consecutive bird passage of the Bridges strain.

Vaccine studies. The vaccine study was composed of four experiments carried out to determine if immunity against infectious synovitis could be produced in chickens by injecting them with infectious material treated with 0.5 percent formalin.

The first vaccine study was carried out with formalinized spleen and liver which had been harvested from birds dying of infection with the Bridges strain. Twenty, 6-week-old, hybrid cockerels were wing banded and divided into groups of 15 and 5 birds. Both groups were maintained in battery brooders and fed a commercial laying ration without antibiotics. Each of the 15 birds received an intramuscular injection of one ml of formalinized material. Twenty-three days later ten of the injected and the five control birds were challenged by an intramuscular injection of one ml of yolk material from embryo infected with the first ten embryo passages of the Bridges strain. The five birds not challenged were set aside for further use.

The second of the vaccine studies was similar to the first except that formalinized yolk instead of tissue vaccine was used. The yolk was from chicken embryos that died of infection with the nineteenth consecutive chicken embryo passage. The yolk was harvested, checked for contamination, pooled and then formalinized. It was stored at 2 C for one week before it was used. Fifteen, 6-week-old, hybrid, male cockerels received one ml of the formalinized yolk by intramuscular injection. They were maintained in battery brooders and fed a commercial laying ration. Twenty-three days after the first injection, ten of the vaccinates and five control birds were challenged by an intramuscular injection of one ml of infectious yolk material. The five birds not challenged were set aside for use in later studies.

The third vaccine study was designed to compare the tissue and yolk vaccines and to detect any additional immunity which might be stimulated by multiple injections of the vaccines. The birds used consisted of five chickens from each of the two preceding experiments and 15 other chickens of the same age, sex, and breeding which had been reared under similar management and nutritional conditions. At the start of the experiment the birds were 9 weeks of age. They were wing banded and divided into four groups among which five controls were distributed. The vaccines were those used in the two preceding experiments and were administered in one ml amounts by the intramuscular route. The four groups were designated A, B, C, and D. Group A consisted of five birds which received two injections of yolk vaccine a month apart. Group B included five birds which received a single dose of the yolk vaccine. Group C consisted of five birds which received an injection of tissue vaccine followed in one month by an injection of yolk vaccine. Group D consisted of five birds which received a single dose of tissue vaccine. In all cases the last injection of vaccine was administered at least two weeks prior to the time of challenge. The birds were challenged by the inoculation of 0.25 ml of liver and spleen suspension into the right foot pad.

In another study, 100 unsexed, broiler type chickens were reared in battery brooders and fed a commercial laying ration until they were 4 weeks of age when they were wing banded and divided into two groups of 50 each. The inoculated birds remained in batteries and the controls were moved to a colony type brooder house. The experimental birds received an intramuscular injection of one ml of a formalinized 50 percent liver and spleen suspension at 2 week intervals until a series of three injections had been administered. Two weeks after the last injection, the inoculated birds were combined with the control birds and placed in a large poultry house. The house had a concrete floor covered with cane litter. During the prior period of separation the control birds became infected with cecal coccidiosis which required the inclusion of sulfaquinoxaline at a level of 0.033 percent in the ration. As many of the con-

trol birds would be carriers of coccidia and the injected birds susceptible because they had been reared on wire, it was necessary that the drug be included in the ration during the remainder of the study. Ten days after the birds were combined, they were challenged by an intramuscular injection of one ml of a 50 percent liver and spleen suspension in nutrient broth. week before it was used. Fifteen, 6-week-old, hybrid, male

Passive immunity. In this study, plasma was collected from the five birds which survived the first natural immunity study. They had been infected once since that time. Approximately 200 ml of plasma were collected and 40 ml inoculated into each of five susceptible birds. Twenty ml were given intraperitoneally and 20 ml were administered intramuscularly in the thigh muscles. Twenty-four hours later, they were combined with an equal number of control birds and the entire group inoculated intramuscularly with one ml of joint exudate suspended in nutrient broth.

Serology. Serum neturalization and serum plate agglutination tests were attempted. The serum neutralization studies were carried out and the results calculated according to the methods described by Reed and Muench.[13] Susceptible hosts for the studies were 6-day-old chicken embryos inoculated by way of the yolk sac.

The plate agglutination test was attempted with equal amounts of antigen and serum. The antigen was prepared by harvesting allantoic fluid as free of particulate matter as possible. This was centrifuged at approximately 3000 rpm for 15 minutes, the supernatant decanted, the sediment resuspended in physiological saline solution and centrifuged again for 15 minutes at 12,500 rpm. The material was removed from the bottom of the tube and diluted 1:10 in physiological saline solution. The yield of antigen was consistently poor and autoagglutination was a problem. The sera used in all studies was collected approximately one month following the recovery of the infected birds.

RESULTS

The first experiment of the natural immunity study was discontinued because of excessive mortality from the experimental infection. The course of antibiotic treatment had little

if any influence on the course of the disease. Twenty-five days after inoculation, only five of the original 50 birds were alive.

During the second experiment of the natural immunity study, 24 of the original 50 birds died from the initial infection in spite of high level antibiotic therapy. Necropsy revealed lesions typical of infectious synovitis and cultures for PPLO and bacteria were essentially negative. All of the surviving birds had swollen footpads, shanks, hocks, and wing joints at the time they were combined with the controls. Because of the presence of lesions in these birds, mortality was the only factor used in evaluating the results of challenge. In the third study of natural immunity, three of the birds inoculated with strain 105 manifested a transient lameness nine days after the initial infection which disappeared after three days. When challenged with the Bridges strain 6 weeks after the initial infection, all of the birds died.

Some protection was apparently conferred on the survivors of an infection with the disease. It is significant that of the 178 birds infected with the Bridges strain, only 23 survived the challenge with the same strain. Five of the birds survived during the first natural immunity study and eighteen in the second. The birds which survived during the first infection of the second study had marked lesions of infectious synovitis. This would indicate that if immunity was obtained, it was only after a severe infection.

There was apparently a complete absence of immunity produced by strain 105 as demonstrated by the loss of 100 percent of the birds following challenge with the Bridges strain. Olson[6] recently described two non-pathogenic strains of infectious synovitis agent which did not stimulate immunity against pathogenic strains. Because strain 105 falls in this category it was not possible to challenge the birds with the homologous strain.

The results of the four vaccine studies are listed in table 1. In the first three studies, only the formalinized tissue vaccine appeared to produce immunity. The fourth study designed to verify these findings in a larger number of birds, failed to reveal demonstrable immunity.

Results of the passive immunity study were inconclusive.

TABLE I

Results of vaccine studies with infectious synovitis

Experiment no.		Number of birds	Number challenged	Number developing evidence of synovitis	Percent Protected
I	Vaccinated with 1 dose tissue vaccine	10	9*	3	66.6
	Controls	5	3*	3	0
II	Vaccinated with 1 dose yolk vaccine	10	10	7	30
	Controls	5	5	2	60
III	Group A (2 doses yolk vaccine)	5	5	3	40
	Group B (1 dose yolk vaccine)	5	5	3	40
	Group C (1 dose tissue vaccine followed by 1 dose yolk vaccine)	5	5	3	40
	Group D (1 dose tissue vaccine)	5	5	2	60
	Controls	5	5	2	60
IV	Vaccinated with 3 doses tissue vaccine	50	50	50	0
	Controls	50	50	50	0

* One immunized and two control birds died of ceccal coccidiosis.

The five birds which received plasma twenty-four hours prior to infection manifested a mild keel bursitis while two of the five controls had lesions typical of a generalized infection with avain infectious synovitis. The other three control birds manifested a bursitis similar to that observed in the serum inoculated birds

Serum neutralization studies did not reveal demonstrable antibodies in the sera of recovered birds or birds that had received formalized vaccine.

Serum plate agglutination tests carried out with the sera of fourteen birds which had survived an attack of avian infectious synovitis were uniformly negative.

SUMMARY

Attempts to produce immunity in chickens by actual infection was carried out in three studies. In one, slight evidence of immunity was demonstrated.

The use of formalinized yolk or liver and spleen suspensions to stimulate immunity in chickens was investigated in four experiments. The results indicated that no practical immunity was produced.

Passive immunity to the disease conferred by the use of sera from convalescent birds was studied with questionable results.

Serum neutralization and plate agglutination studies were carried out with sera from birds which had survived the disease or been injected with vaccine. Significant antibody levels were not detected.

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THE INFLUENCE OF DIETARY CALCIUM-PHOSPHORUS AND TEREPHTHALIC ACID ON ANTIBIOTIC CONTROL OF EXPERIMENTAL INFECTIOUS SYNOVITIS

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A NUMBER of experimental investigations have dealt with ability of feed-administered drugs to control infectious synovitis. Oxytetracycline* (OTC) and chlortetracycline** (CTC) have consistently been found effective for this purpose.[6,8,9,13] Furazolidone has been reported active in some trials[1,2] but less effective in others.[7,14] Under field conditions, orally administered antibiotics, while generally effective at recommended use levels, do not always provide complete control of severe outbreaks. Increasing the therapeutic

* Terramycin, Chas. Pfizer & Co., Inc.
** Aureomycin, Lederle Laboratories Division, American Cyanamid Co.

effectiveness by raising antibiotic concentrations in the feed has not generally proven to be economically feasible. Hence a real need exists for improving efficacy of currently used antibiotic treatments.

When it was observed that serum concentrations of orally administered oxytetracycline could be "potentiated" or enhanced by alteration of dietary calcium and phosphorus or by feeding terephthalic acid[4,12] it seemed of interest to investigate the influence of these potentiation techniques on antibiotic control of infectious synovitis.

EXPERIMENTAL PROCEDURE

Experiment 1.—This trial originally was designed to evaluate the prophylactic effect on infectious synovitis (IS) of oxytetracycline, and chlortetracycline at 200 gm per ton of a practical broiler ration. As a result of the antibiotic blood serum increases noted with terephthalic acid in previous experiments, two additional treatments were evaluated: OTC at 100 gm per ton and OTC at 100 gm per ton plus 0.5 percent terephthalic acid (TA). Each treatment group as well as the infected and non-infected controls comprised 24 or 48 3-week-old Nichols White Cross birds. Chicks were individually weighed and allotted on an equal sex basis into two or four replicate groups of 12 birds each.

Designated lots were then infected with the IS agent (isolated from a field case in Tennessee) by injection of 0.25 ml of infectious synovial exudate suspended in saline into one foot pad. Birds were immediately placed on the medicated rations. Individual weights were taken and lesions recorded at 1, 2 and 3 weeks post-infection. Lesions were scored by means of the following arbitrary system: 0 = no lesion; 1 = questionable; 2 = marked; 3 = severe. Mortality records were also maintained.

Experiment 2.—This was a determination of the influence of TA at various levels on the prophylaxis of IS with 100 gm/ton OTC in the commercial feed supplement form. TA was incorporated in the practical broiler diet at levels ranging from 0.1 to 0.5 percent. A total of 340 4-week old White Rock chicks were allotted into eight groups of 40

birds and one group of 20 birds, the last serving as the infected control. All groups had equal numbers of males and females, and were of comparable average weight. In addition to infected and non-infected controls, one infected group received OTC at 200 gm/ton with no TA.

Birds were injected with the IS agent as described in experiment 1, and immediately placed on the medicated diets. Birds were individually weighed and lesions recorded at 2, 3 and 4 weeks post-infection. Lesions were scored as described for experiment 1. Mortality was recorded daily.

Experiment 3.—This was an evaluation of the influence of OTC and CTC on IS when administered in a diet with altered calcium-phosphorus levels and containing 0.25 percent TA. The logic for altering the levels of dietary calcium and phosphorus was based on findings that OTC blood serum concentrations were markedly increased by reducing the Ca content of the ration, or by increasing phosphorus above the "normal" level.[12] The concentrations of calcium and phosphorus chosen for the experimental diet were 0.7 and 2.08 percent, respectively.

Because of insufficient birds, treatment groups measuring effect of mineral alterations only or TA supplementation only were not included. Infected and non-infected control groups were maintained on the practical broiler diet (Ca, 1.2 percent; P, 0.7 percent). A non-infected control on the experimental diet was included to determine its effect on growth .

A total of 110, 4-week-old Nichols White Cross birds were individually weighed and allotted into 11 groups of equal weight, each comprised of five males and five females. The birds were infected as described for experiment 1, except that IS strain 29-M* was used, and then were placed immediately on the treatments. Individual weights and lesion scores were recorded at 7, 14 and 24 days post-infection. Lesion severity was estimated and scored according to the following system: 0 = no lesion; 1 = a questionable or slight swelling of the foot pad; 2 = a marked swelling of

* Obtained from Dr. N. O. Olson, University of West Virginia.

the foot pad; 3 = swelling of the foot pad and shank; 4 = same as 3 plus extension to hock. Daily mortality records were maintained.

Experiment 4.—The prophylactic effect of OTC as the commercial feed supplement at 100 and 200 gm/ton was determined in this experiment. Antibiotic at the indicated levels was incorporated into both a practical corn-soy diet containing 1.18 percent calcium and 0.7 percent phosphorus and a similar diet containing only 0.18 percent calcium and 0.7 percent phosphorus (P added as $Na_2 HPO_4$). All antibiotic treatments were evaluated, both in the presence and absence of TA supplementation (0.375 percent). Infected and non-infected controls were maintained on the practical diet. An additional non-infected control on the low-Ca diet was employed in order to show the influence of dietary mineral alterations on growth. A total of 110, 7-week-old Nichols White Cross birds were allotted into 12 groups of equal weight with 10 birds each, five of each sex. Birds were infected with the Wills CE-5 strain of IS by injection of 0.5 ml of infectious synovial exudate suspended in sterile serum into one foot pad of each bird. Non-infected controls were injected with a suspension of non-infectious foot pad tissue. All birds were then placed on the appropriate medicated rations. Birds were individually weighed at 8, 14, 21 and 28 days, and lesion severity was determined at 8, 10, 14, 15, 21 and 28 days. The 0 to 9 scoring system recommended by the West Virginia workers[8] was used.

One week post-exposure blood samples were obtained by wing vein puncture from five birds in each treatment group (three males, two females). Sera were harvested and assayed for antibiotic content according to the procedures outlined by Grove and Randall.[3] At the completion of the 28-day trial, bone ash and bone calcium determinations were made on the foot bones of representative birds.

Experiment 5.—The relative therapeutic efficacy on experimental IS of OTC as the commercial feed supplement when incorporated at 200 gm/ton in a practical broiler ration (Ca, 0.93 percent; P, 0.8 percent) and in a similar "low calcium" diet (Ca, 0.47 percent; P, 0.8 percent) was determined

in this experiment. The effect of supplementing each of these rations with 0.375 percent TA was also evaluated. Due to space limitation in this floor pen trial, the only control was an infected group receiving the practical ration. Sixty 5-week-old Nichols White Cross birds (30 males and 30 females) were employed in each treatment group. Birds were so allotted that average group weights were comparable. Injection of the Wills CE-5 IS strain was carried out as in experiment 4. All the birds were maintained on a practical, non-medicated ration at the time of exposure to IS and for 4 days following exposure. At this time, birds were placed on the treatments. Individual weights were recorded at 10, 17, 24 and 31 days, while lesion severity was estimated at 4, 10, 17, 24 and 31 days post-infection. The lesion scoring system employed for experiment 4 was utilized here.

One week after being placed on medicated feed, six males and six females were selected at random from each treatment group and bled from the wing vein. Sera were harvested and assayed for OTC content by the method previously cited. Other data recorded at the conclusion of the trial were: total feed consumption and bone ash and bone Ca values.

RESULTS

Effect of oxytetracycline (OTC) and chlortetracycline (CTC) on IS produced by injection of a Tennessee strain of virus is shown in table 1. The severity of the experimental infection is attested by the high mortality rate (50 percent) and extreme debility of all surviving birds in the infected control group. At the 200 gm/ton level, both OTC and CTC adequately controlled mortality and lesion development. In addition, average weight gains with both antibiotics were comparable to those of the non-infected control. No appreciable difference in efficacy was shown for these antibiotics. OTC at the 100 gm/ton level, while adequately controlling mortality and permitting good weight gains, failed to satisfactorily suppress lesion development. However, when 0.5 percent terephthalic acid (TA) was incorporated in the ration, the same antibiotic level gave disease control comparable to

TABLE I

Influence of antibiotics in the ration on the clinical course of experimentally-induced infectious synovitis

Treatment	Mortality No. dead/ No. started	Av. 3-wk. weight gains (gm)	Lesion distribution in survivors (3 weeks) 0	1	2	3	Percent saleable birds#
Infected control	12/24	106	0	0	0	12	0
Non-infected control	2/48†	425	46	0	0	0	96
OTC* 200 gm/ton	0/48	444	39	7	2	0	96
CTC** 200 gm/ton	1/48	447	38	9	0	0	100
OTC 100 gm/ton	1/48	399	20	15	9	3	73
OTC 100 gm/ton + 0.5% TAx	0/24	459	20	4	0	0	100

† Death due to non-specific causes
# Birds with lesion score of 1 or less
* Oxytetracycline (as TM-10)
** Chlortetracycline (as Aurofac-10)
x Terephthalic acid

that obtained at the 200 gm/ton level of OTC or CTC without TA.

The second experiment was designed to measure the effect of supplementing the ration with graded levels of TA on OTC efficacy in experimental IS. Results are presented in table 2.

The virulence of the Tennessee IS strain had been intensified by several successive passages in birds. In this trial, 100 percent of the infected control birds died within two weeks after infection. OTC at 200 gm/ton, which in the previous trial had provided nearly complete control of the disease, failed to satisfactorily protect the birds as evidenced by reduced weight gains and by the greater number of birds with lesion scores of 2 and 3. The 100 gm/ton level of antibiotic modified the clinical severity of the disease only slightly, as evidenced by very poor weight gains and a high mortality rate (62.5 percent). Addition of TA at levels of 0.1 percent or 0.2 percent had a very favorable influence on mortality, lesion scores, and weight gains, but appeared to be suboptimum since nearly complete control was obtained at TA levels ranging from 0.3 percent to 0.5 percent. A plateauing of activity was very apparent at TA levels in this range as evidenced by similarity of values for weight gain and percent saleable birds. On the basis of the same criteria, these three treatments gave results that were slightly superior to those obtained at the 200 gm/ton level of antibiotic without TA.

The influence of 100 and 200 gm/ton levels of OTC and CTC on experimental IS induced by injection of the 29-M strain of virus was determined in experiment 3. This comparatively mild strain did not produce mortality in any group. Treatment was administered both in a practical broiler diet (PB) with 1.2 percent Ca and 0.7 percent P, and in an experimental diet (EX) containing 0.7 percent Ca, 2.08 percent P, and 0.25 percent TA.

Since birds on diet EX developed a marked diarrhea (probably due to high dietary Na and P) which prevented satisfactory gains, it was replaced one week post-infection by a practical diet (Ca 1.2 percent; P, 0.7 percent) containing

TABLE II

Prophylaxis of experimental infectious synovitis by oral oxytetracycline as influenced by various levels of dietary terephthalic acid

Treatment	Mortality No. dead/ No. started	Av. 4-wk. weight gains (gm)	Lesion distribution in survivors (4 weeks)				Percent saleable birds#
			0	1	2	3	
Infected control	20/20	—	—	—	—	—	0
Non-infected control†	0/40	647	0	0	0	0	100
OTC* 200 gm/ton	1/40	608	16	13	9	1	73
OTC 100 gm/ton	25/40	372	4	2	1	8	15
OTC 100 gm/ton + TA** 0.1%	10/40	538	8	9	4	9	43
OTC 100 gm/ton + TA 0.2%	3/40	586	18	12	4	3	75
OTC 100 gm/ton + TA 0.3%	0/39	638	26	10	2	1	92
OTC 100 gm/ton + TA 0.4%	1/40	636	23	15	1	0	95
OTC 100 gm/ton + TA 0.5%	1/37	620	20	16	0	0	97

† Ration contained oxytetracycline as TM-10 (100 gm/ton)
# Birds with lesion score of 1 or less
* Oxytetracycline as TM-10
** Terephthalic acid

no antibiotic or TA. Birds on diet PB received the medicated rations for the entire 3 week test period. Average lesion scores and weight gains are shown in table 3.

Lesion scores of birds in the infected control group ranged from 2.6 to 3.0 on the 0-4 scale during the 3 week test period. Only one bird had definite extension to the hock. These birds had weight gains averaging 215 grams less than those of the non-infected control on the PB diet. Weight gains (0 to 3 weeks) of birds in the non-infected control group on the EX diet averaged approximately 100 gm less than those of the birds with the PB diet. However, during the 1 to 3 week period, gains were comparable for both groups. The 100 gm/ton level of both OTC and CTC gave markedly better control of IS lesions on the EX diet than on the PB diet, although the antibiotic was administered for only one week instead of three. It can also be noted that the 0 to 3 week weight gains were not appreciably different for birds on the two diets, despite the fact that gains were much lower on the EX diet during the one week medication period. This is explained by the higher weight gain of birds on EX diet between 1 to 3 weeks, which probably resulted from better disease control. At the 200 gm/ton level, both antibiotics suppressed lesion development so well that no appreciable improvement due to the potentiated diet could be demonstrated.

The 0 to 3 week weight gains of infected birds on the EX diet containing antibiotic were as good as those of the non-infected control on this diet in every instance, and with one exception were actually higher than the control.

The influence of dietary calcium and terephthalic acid on OTC activity aginst IS produced by artificial infection of birds with the Wills CE-5 strain of virus, was studied in experiment 4. Since most of the medicated diets appeared to have provided nearly complete suppression of lesion development at the 10th day post-infection, it was decided to withdraw all treatment at that time. All birds were then placed on a diet containing Ca at 1.18 percent and no TA or antibiotic. It was hoped that this change in the original experimental design would allow clinical IS to develop in birds that had received

TABLE III

Control of infectious synovitis by oxytetracycline and chlortetracycline as influenced by an experimental diet containing terephthalic acid

Treatment	Diet	Av. lesion score (weeks post-inoculation)			Average wt. gains, gm	
		1	2	3	(0 - 3 wk.)	(1 - 3 wk.)
Infected control	PB*	2.8	3.0	2.6	494	417
Non-infected control	PB	0.0	0.0	0.0	709	581
	EX**	0.0	0.0	0.0	602	552
OTC# - 100 gm/ton	PB	1.2	1.7	0.9	668	494
	EX	0.0	0.2	0.2	625	538
CTC## - 100 gm/ton	PB	0.6	1.4	0.7	652	499
	EX	0.8	0.4	0.0	681	554
OTC - 200 gm/ton	PB	0.3	0.5	0.3	661	497
	EX	0.0	0.0	0.0	596	504
CTC - 200 gm/ton	PB	0.0	0.2	0.1	648	493
	EX	0.2	0.2	0.2	654	548

* Practical broiler diet (Ca 1.2%; P, 0.7%)
** Experimental diet (Ca, 0.7%; P, 2.08% and terephthalic acid 0.25%)
# As oxytetracycline HC1
As chlortetracycline HC1

inadequate treatment. The 0 to 8 day weight gains which were made during the medication period, and the 0 to 28 day gains which represent those of the entire test period, are shown in table 4. Mortality and antibiotic blood serum level data are also shown.

In the infected control group, six of 10 birds died, and the survivors lost an average of 50 grams, showing that the exmental infection was quite severe.

The weight gains of the non-infected control groups indicate that lowering dietary calcium from 1.18 percent to 0.18 percent for a 10-day period suppressed growth. The fact that the difference in weight gain between the two controls was not appreciably greater at 28 days than at 8 days indicates that birds taken off the low Ca diet at 10 days grew at approximately the same rate after this time as did those which had been maintained on the higher calcium level for the entire 28 day period.

All antibiotic treatments, except possibly the 100 gm/ton level of OTC in the 1.18 percent Ca diet, permitted 8 day weight gains that were comparable to those attained by the "normal" Ca control group. The fact that such excellent gains were obtained on the low-Ca diets containing antibiotic suggests a possible sparing effect of OTC upon Ca. A similar phenomenon has been reported for CTC[5].

Examination of the 0 to 28 day gains shows that none of the 10-day treatments completely eliminated the disease, as evidenced by the fact that all groups failed to maintain weight gains equivalent to those of the non-infected high Ca control. However, weight gains in the low Ca treatment group receiving OTC at 200 gm/ton and TA at 0.375 percent were comparable to those obtained in the low-calcium non-infected control group. In general, TA supplementation favorably affected gain regardless of type of ration or antibiotic level. One of 10 birds died in each of the groups on 100 gm/ton OTC, except the group on the low calcium diet receiving TA.

It is evident that antibiotic blood serum levels could be markedly increased, either by employing a low level of dietary calcium, or by TA supplementation. Use of both potentiating

TABLE IV

Prophylactic effect of oxytetracycline on experimentally-induced infectious synovitis as influenced by calcium and terephthalic acid

Treatment	Dietary Ca. %	Mortality No. dead/ No. started	Weight gains (gm) (0- 8 days)	Weight gains (gm) (0 - 28 days)	OTC serum levels (mcg/ml)
Infected control	1.18	6/10	10	–50	<0.15
Non-infected control	1.18	0/10	279	819	<0.15
	0.18	0/10	220	711	<0.15
OTC* 100 gm/ton	1.18	1/10	245	567	<0.15
	0.18	1/10	280	524	0.295
OTC 100 gm/ton + 0.375%TA #	1.18	1/10	273	587	0.252
	0.18	0/10	280	596	0.488
OTC 200 gm/ton	1.18	0/10	266	577	0.235
	0.18	0/10	285	638	0.612
OTC 20 gm/ton + 0.375% TA	1.18	0/10	302	669	0.412
	0.18	0/10	271	699	0.812

* Oxytetracycline (as TM-10)
\# Terephthalic acid

techniques simultaneously gave nearly a four-fold increase in antibiotic serum levels. This suggested additivity of the two methods, since each approximately doubled serum levels when used alone. This finding is consistent with those previously reported by the authors.[10,11]

Average lesion scores of birds on the 1.18 percent calcium treatments are plotted versus time in figure 1. It can be observed that a rapid rise in lesion count occurred in the infected control group. By the tenth day, it averaged 6.8, and although somewhat variable, due to the high mortality rate in this group, stayed at a high level. Relatively poor IS control was obtained with the 100 gm/ton level of OTC, as evidenced by the lesion score of 4.7 after 10 days medication. OTC at 200 gm/ton gave better control during the medication period, and it seems likely that if medication had not been stopped, adequate suppression of lesions would have continued. Lesion scores of 2 or less are considered adequate control, since it is doubtful if birds with such minor lesions would be downgraded. Use of 0.375 percent TA with 100 gm OTC/ton gave results comparable to those obtained at the 200 gm ton level of antibiotic alone. TA supplementation at the higher antibiotic level suppressed lesions for an additional four day period after removal of medication.

Average lesion scores of the birds on the low-calcium diets are similarly plotted in figure 2. Here, in contrast to results obtained on the 1.18 Ca diet, OTC at 100 gm/ton adequately controlled lesions during the medication period, as did OTC at 200 gm/ton. However, 200 gm/ton maintained a lesion score below 2 for an additional 4 days after medication was stopped. Addition of TA to diets containing 100 gm and 200 gm/ton of antibiotic resulted in adequate lesion suppression for 5 and 12 days, respectively, beyond the medication period.

In general, blood serum concentrations correlated well with control of lesions. With OTC serum concentrations below the minimal detectable quantity (0.15 mcg/ml) adequate control of lesions was not obtained, even during the medication period. At levels between 0.15 and 0.3 mcg/ml, con-

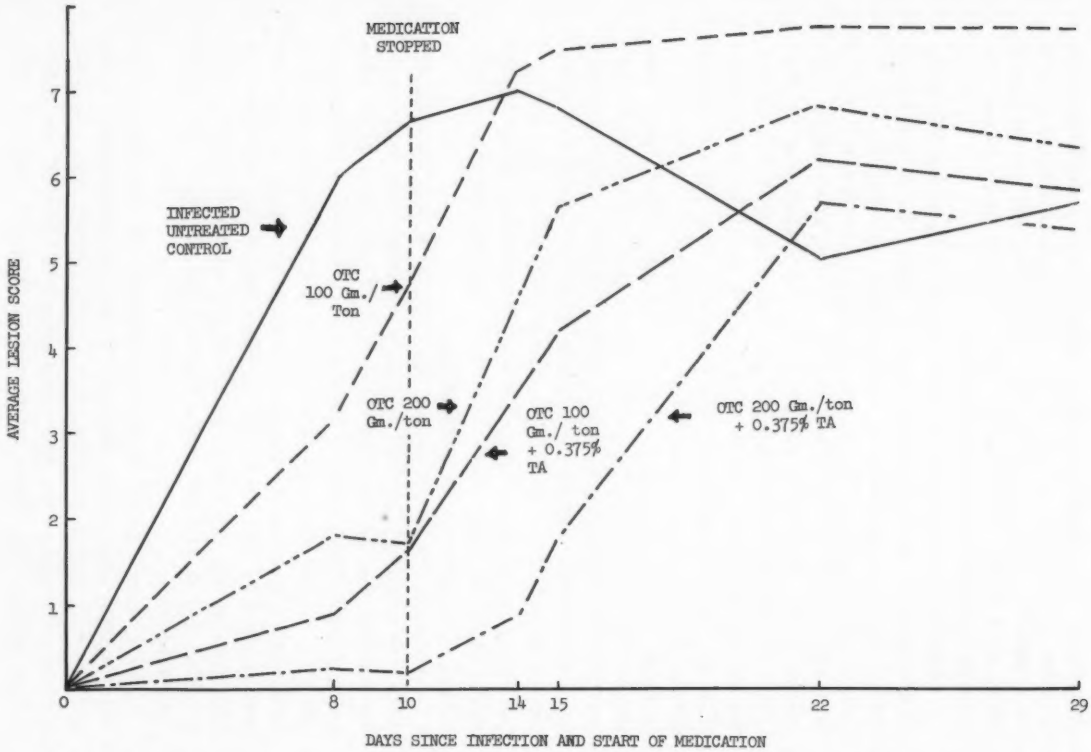

Fig. 1. Lesion scores of synovitis in chicks as influenced by oxytetracycline or oxytetracycline plus terephthalic acid administered simultaneouslv with infection. Normal calcium diet (1.18%).

trol was obtained during the medication period only; levels in excess of 0.4 mcg/ml provided control that extended beyond the medication period. Duration of lesion control after withdrawal of medication correlated well with the antibiotic serum level, except at the 200 gm/ton level of OTC on the low Ca diet. Here an average serum level of 0.612 mcg/ml. did not give the same protection as OTC at 100 gm/ton plus TA, where there was an average serum level of 0.488 mcg/ml.

It is of interest that no evidence of rickets or differences in bone ash and bone calcium were found at 28 days for birds previously maintained on rations containing 1.18 and 0.18 percent calcium for 10 days. For birds on the "normal" calcium diet, ash and Ca were 45.34 and 20.85 percent respectively, and corresponding values with the low calcium diet were 46.30 and 20.98 percent.

Experiment 5 was a therapeutic trial in which medication was withheld until four days after infection of birds with the Wills CE-5 strain of IS. OTC was administered at 200 gm/ton in diets containing 0.93 and 0.47 percent calcium in the presence and absence of 0.375 percent TA. Average weight gains, lesion scores, antibiotic blood serum concentrations, mortality, and the percent saleable birds are shown in table 5.

The Wills CE-5 strain maintained its extreme virulence in this trial as evidenced by the high mortality rate and poor gain of surviving birds in the infected control group. None of the survivors in this group was saleable. All treatments markedly reduced mortality and permitted good weight gains. Addition of TA to the diet resulted in increased weight gains on both high and low-calcium diets. It is interesting that birds maintained for one month on a diet having a calcium level of only 0.47 percent had gains only slightly inferior to those of birds on a diet containing 0.93 percent calcium.

At the time of treatment, the average lesion scores for the various groups ranged from 2.4 to 3.6. All of the groups receiving antibiotic treatment showed a marked recession in lesions by 27 days post treatment, while those of the infected control became more severe, even though only the less seriously affected birds survived to this time.

phthalic acid administered simultaneously with infection. Normal calcium diet (1.18%).

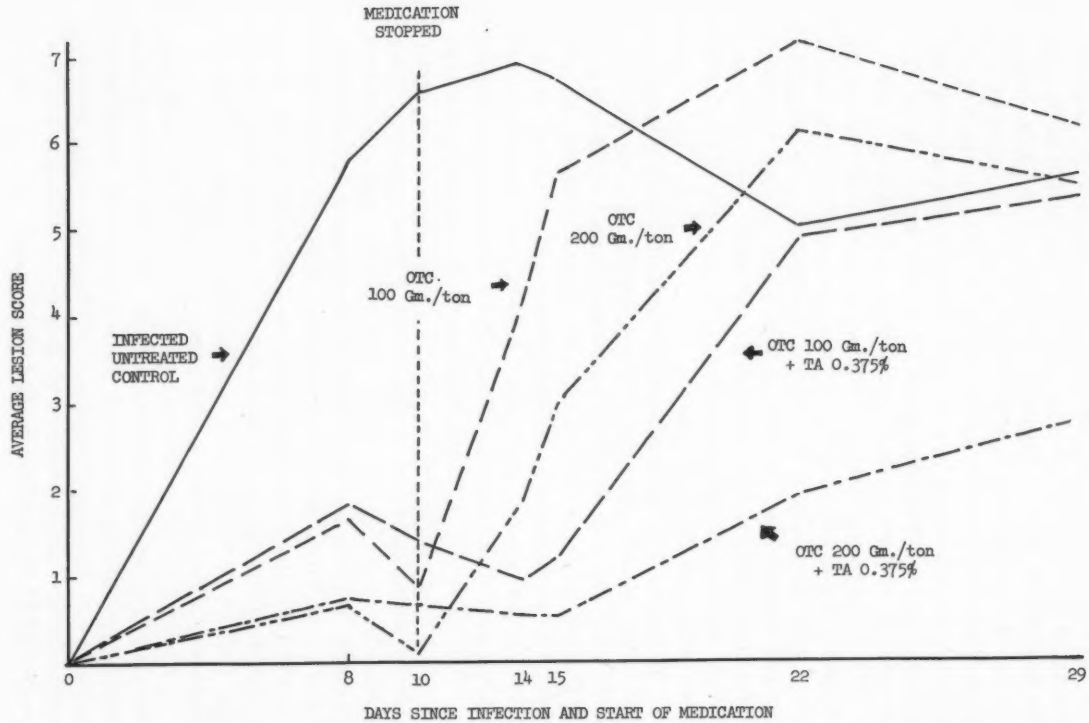

Fig. 2. Lesion scores of synovitis in chicks fed oxytetracycline or oxytetracycline plus terephthalic acid simultaneously with infection. Low calcium diet (0.18%).

The excellent therapeutic action of the 200 gm/ton OTC treatment in the 0.97 percent Ca diet would not permit the possible benefits of altering dietary Ca or TA supplementation to be manifested to any appreciable extent. This suggestion is borne out when one compares the percent saleable birds in the various treatment groups. The range for all treatments was found to be 87 to 95 percent.

The OTC serum concentration of 0.291 mcg/ml in birds on 200 gm/ton OTC in diet containing 0.93 percent Ca was apparently sufficient to control the disease. It is of interest that while TA supplementation markedly enhanced antibiotic serum levels, reduction of dietary Ca from 0.93 to 0.47 percent resulted in only slightly increased OTC serum levels.

Average lesion scores for birds of experiment 5 are plotted versus time in figure 3, in order to show the rate of lesion regression in the various treatment groups.

The infected, untreated control had high average lesion scores at all measurement periods, although they were variable because of high mortality. All of the groups potentiated either by removal of calcium, addition of TA or both, showed a reduction of lesion score on the 6th day. However, in the group receiving 200 gm per ton of oxytetracycline without potentiation, the lesion score was slightly increased at 6 days. If this constitutes a real difference, it indicates a possible benefit of potentiating the antibiotic. One week later, the average score on all treatments had dropped to less than 2. Subsequently, a slow reduction in the already minimal lesion score occurred in the treated groups.

Bone ash and bone calcium determinations made on toe bones after 27 days on the 0.93 and 0.47 percent calcium diets, showed that, while bone ash values were comparable (42.99 and 42.85 percent respectively), calcium values for birds on the 0.47 percent calcium diet (20.76 percent) were somewhat lower than those of birds on the 0.93% calcium diet (21.85 percent).

No appreciable differences in feed consumption were noted for the various antibiotic treatment groups.

Fig. 2. Lesion scores of synovitis in chicks [illegible] multaneously with infection. Low calcium diet (0.18%).

TABLE V

The influence of dietary calcium and terephthalic acid on the therapeutic efficacy of oxytetracycline in infectious synovitis

Treatment	Dietary Ca. %	Weight gains (gm)	Mortality No. dead/ No .started	Av. lesion score		Percent# Saleable Birds	OTC serum conc. (mcg/ml)
				Time of treament	27 days post treament		
Infected control	0.93	64	45/60	3.5	4.5	0	<0.15
OTC* 200 gm/ton	0.93	837	4/60	3.1	0.5	90	0.291
OTC 200 gm/ton + 0.375% TA**	0.93	906	4/60	3.6	0.4	93	0.507
OTC 200 gm/ton	0.47	792	5/60	2.4	0.5	87	0.343
OTC 200 gm/ton + 0.375% TA	0.47	853	1/60	3.4	0.4	95	0.577

* Oxytetracycline (as TM-10)
\# Birds with lesion score of 2 or less.
** Terephthalic acid

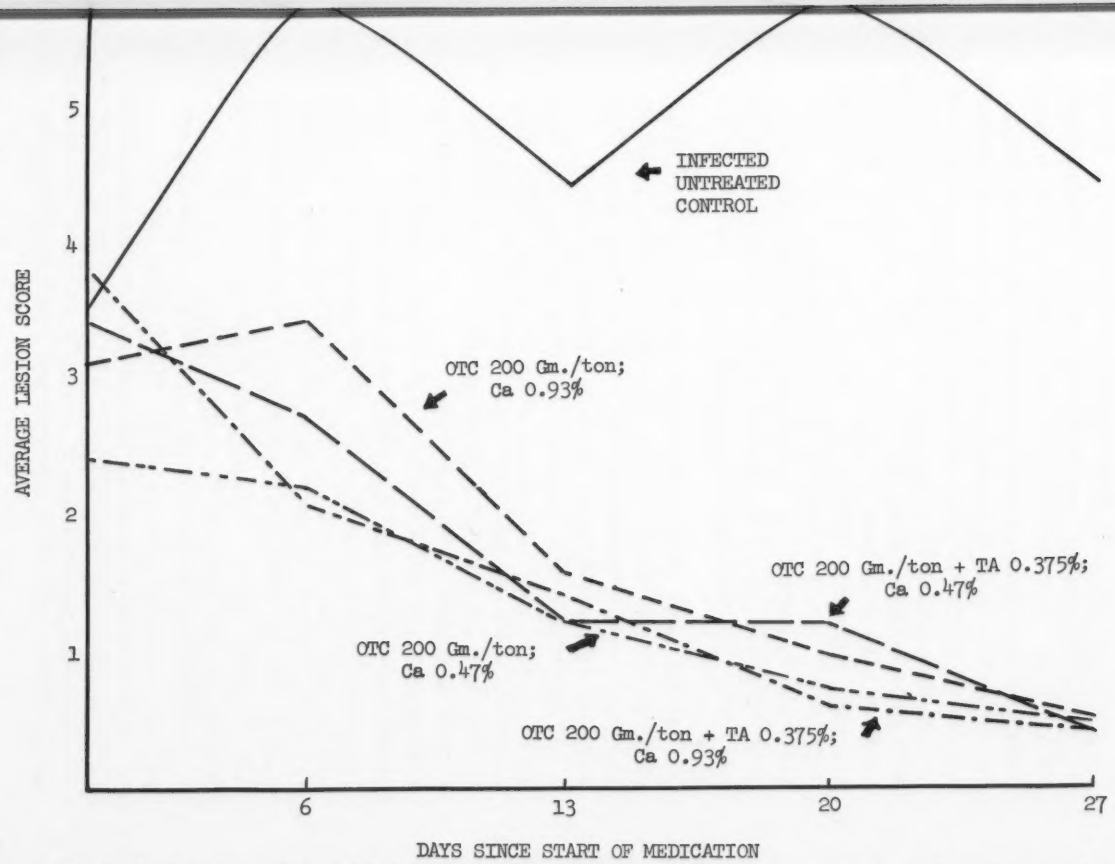

Fig. 3. Lesion scores of synovitis in chicks as affected by oxytetracycline or oxytetracycline plus terephthalic acid administered 4 days after infection.

DISCUSSION

Data given in the preceeding experiments show that potentiation of orally administered antibiotics by a temporary reduction in dietary calcium, or addition of compounds such as terephthalic acid (TA) may have a place in the control of infectious synovitis (IS). Although there is a setback in weight gains accompanying removal of dietary calcium for extended periods of time (10 days was the minimum period studied), the likelihood of getting more birds to market through enhancing therapeutic effectiveness of antibiotic medication may warrant use of this technique. The possibility also exists that, in the usually milder, naturally occurring outbreaks of IS, a shorter treatment period may be sufficient, thus reducing loss in weight due to inadequate calcium.

On the basis of antibiotic blood serum level data in experiment 5, there would appear to be limited benefit in the Ca reduction method of potentiation if the dietary level is not reduced below 0.5 percent during the treatment period.

Terephthalic acid, in these experiments, has not been found to have any detrimental effect on weight gains, while markedly improving control of experimental IS. The optimum level of terephthalic acid to be employed in conjunction with OTC prophylaxis or therapy would seem to be somewhere between 0.3 and 0.4 percent of the ration. Data in these experiments show that TA supplementation in this range increases the effectiveness of the 100 gm/ton level of oxytetracycline more than two-fold. Use of this acid in a diet low in calcium (0.18 percent) further improved the effectiveness of OTC in combatting experimental IS., undoubtedly as a result of the marked enhancement in antibiotic serum levels induced by application of these procedures.

Response of artificially induced IS to OTC appeared to be directly correlated with antibiotic blood serum level.

Terephthalic acid, particularly when administered in low Ca diets, may offer the following advantages in IS control: (1) Improved performance of presently employed antibiotic levels in terms of clinical response and duration of treatment,

and (2) satisfactory control of IS at antibiotic levels that are currently borderline or minimal in effect with the resulting economic benefit.

These antibiotic potentiation techniques may have a similar place in controlling other systemic diseases of poultry. Since these techniques influence antibiotic blood serum levels rather than the disease agent itself, it is doubtful whether they would have application where dietary antibiotic levels below 100 gm/ton are being employed for treatment.

ACKNOWLEDGMENT

Experiments 1 and 2 of this report were conducted under the supervision of Dr. E. H. Peterson, now at the University of Arkansas.

SUMMARY

1. Addition of terephthalic acid to poultry diets markedly enhanced the prophylactic effect of dietary oxytetracycline on experimental infectious synovitis.
2. The optimum terephthalic acid level with 100 gm/ton of antibiotic appeared to be between 0.3 and 0.4 percent of the ration.
3. A similar enhancement in control of infectious synovitis by oxytetracycline was achieved by reducing the dietary calcium level to 0.18 percent during the treatment period.
4. Simultaneous application of both antibiotic potentiation methods resulted in an additive increase in oxytetracycline efficacy.
5. Response of a severe infectious synovitis infection to therapy was closely correlated with the antibiotic blood serum concentrations produced by the various treatments.

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THE INFLUENCE OF TEREPHTHALIC ACID ON OXYTETRACYCLINE SERUM LEVELS IN CHICKENS. STUDIES N MODE OF ACTION. I.

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THE authors have shown[5] that the increased oxytetracycline* serum levels produced in poultry by simultaneous administration of antibiotic and terephthalic acid (TA) via the ligated duodenal loop are apparently unrelated to inhibition of antibiotic absorption by calcium. This is in contrast to citric acid and sodium metaphosphate which are capable of increasing serum levels of oxytetracycline (OTC) only when intestinal absorption of the antibiotic is suppressed by cations such as calcium. They also noted that a depression in urine antibiotic concentrations due to terephthalic acid occurred concurrently with increased antibiotic serum and liver levels. This finding suggested that the higher antibiotic levels in the presence of terephthalic acid may have occurred as a result of an alteration in the renal excretion rate of oxytetracycline. Although no such compound has been demonstrated to produce this effect with tetracycline antibiotics, others (probenecid**, carinamide, p-aminohippuric acid) have been reported to suppress renal excretion of penicillin.[2] It is of interest that these drugs show some structural similarities to terephthalic acid.

* The trade mark of Chas. Pfizer & Co., Inc. for oxytetracycline is Terramycin.

** The trade mark of Sharp & Dohme Division, Merck & Co., Inc. for probenecid is Benemid.

The purpose of the present study was to obtain additional information concerning the manner in which terephthalic acid influences oxtetracycline serum levels in chickens.

EXPERIMENTAL PROCEDURE

Experiment 1. The effect of terephthalic acid on total urine output was determined in five separate trials. In the first three trials, OTC and TA were simultaneously administered into the ligated duodenal loop according to a method previously described.[5] In the others, OTC was given intramuscularly and TA subcutaneously. In each trial the average urine output for 5 birds* receiving 4 mg OTC + 66.4 or 132.8 mg terephthalic acid was compared with that of 5 birds receiving 4 mg OTC only. This was accomplished by maintaining the birds in individual units during the 4 hour test period. Each unit had a metal ½ inch mesh floor over a galvanized metal bottom that sloped toward a central aperture. Collection was made into small beakers placed directly below the aperture. Fecal contamination of urine was prevented by plugging the rectum with a cotton wad. Urine samples for assay were collected at pre-designated sampling times by insertion of a small speculum into the cloaca. The quantities of urine acquired by this procedure were measured and the resulting values added to that obtained by collection in the beaker to give total urine output. The desirability of collecting urine specimens for assay at pre-designated times has been previously demonstrated.[5]

Experiment 2. The ligated duodenal loop procedure was employed in this experiment to determine the effect of 0.4 millimole (66.4 mg) terephthalic acid, 0.4 millimole (71.7 mg) glucosamine HCl alone and in combination on serum, liver and urine levels following a 4 mg dose of OTC. Tests were conducted both in the presence and absence of 0.4 millimole (16 mg) added calcium. Forty 7 and 8-week-old birds having an average weight of 1190 gm (range from 940 to 1325) were divided into eight equally weighted groups and given the various treatments by injection into the loop in 4.0 ml volume. Birds did not have access to food for approximately 18 hours

* All birds in this study were Nichols White Cross.

prior to injection of the test solutions into the duodenal loops, but did have water available until 2 hours pre-injection. Two and 4 hour post-injection blood and urine sample collections were made. After 4 hour samples had been taken, birds were sacrificed and the liver recovered. All samples were handled as previously described[1] and assayed for antibiotic content by the cyclinder-plate diffusion method employing *Bacillus cereus* var. *mycoides* (ATCC 9634) as the test organism. Anhydrous OTC base was used as the standard of comparison. All values were corrected and are reported as OTC HCl activity.

Experiment 3. A total of 35 eight-week-old birds were employed in this experiment to determine the effect of terephthalic acid and sodium terephthalate, administered by various routes, on blood serum, urine and liver antibiotic concentrations following intramuscular (IM) injection of 4 mg oxytetracycline in 2.0 ml volume. Terephthalic acid (66.4 mg in 2.0 ml volume) was administered subcutaneously (SQ) while 84 mg Na terephthalate was given by SQ, IM or intravenous (IV) routes. The 35 birds (average weight 1230 gm, range 1190-1350) were divided into seven equally weighted groups and fasted prior to treatment, as described for the preceding experiment. The 2 and 4 hour blood serum and urine specimens and the liver samples were assayed by the method indicated in experiment 2.

Experiment 4. The effect of 168 mg SQ-administered sodium terephthalate on blood serum levels following a 4 mg IM dose of OTC was determined over a 6 hour test period. All injections were given in 2.0 ml volume. A total of 24 eight-week-old birds averaging 1237 gm (range 1072-1302) were divided into two equally weighted groups. The OTC dose was administered to both groups, while only one received sodium terephthalate. Blood samples for assay were collected at 2, 4 and 6 hours post-injection.

Experiment 5. The effect of sodium terephthalate on antibiotic levels when administered after no further OTC absorption could take place was determined. A total of 36 eight-week-old birds ranging in weight from 1070 to 1270 gm (average 1145) were employed. An 8 mg dose of OTC was in-

jected into the ligated duodenal loop of all birds. One hour post-injection, the loop was re-exposed and the major blood vessels supplying the ligated portion tied off (no. 2 silk) approximately one cm anterior to the gut ligatures. An additional ligature was applied to the entire loop just anterior to the one closing off the blood vessels. That this procedure adequately stops blood circulation and hence absorption was evidenced by cyanosis of duodenal loops a short time after treatment. In addition, antibiotic injected into a loop previously ligated in this manner could not be detected in serum or urine specimens at any time post-injection. Immediately following application of ligatures, blood samples were taken for determination of OTC concentrations at the time absorption was stopped. Sodium terephthalate (84 mg) was then administered subcutaneously in 2.0 ml volume to one-half the birds. Blood samples were collected at 1, 2 and 3 hours after the blood vessels were ligated. All serum specimens were assayed for antibiotic content.

Experiment 6. The influence of several isomers and analogs of terephthalic acid on OTC serum, urine and liver levels was investigated. Forty 8-week-old birds with an average weight of 1163 gm (range 1100-1235) were allotted into eight equally weighted groups of 5 birds. There were eight treatments as follows: 4 mg OTC alone, 4 mg OTC plus respectively terephthalic acid, iso-phthalic acid, phthalic acid, benzoic acid, dimethylterephthalate, probenecid (p-dipropylsulfamyl-benzoic acid), or p-hydroxybenzoic acid, each at the 0.4 millimole level. All compounds were administered into the duodenal loop. Blood serum and urine specimens for assay were collected 2 and 4 hours post-injection. Liver specimens were obtained at the conclusion of the trial (4 hours).

Statistical examination of data from these experiments was conducted whenever applicable by analysis of variance. The use of the term "significant" refers to statistical significance at probability level for error of 5 percent or less.

RESULTS AND DISCUSSION

Data of five individual trials of experiment 1 are sum-

marized in table 1 and show that no change in urinary volume was caused by administration of TA up to 132.8 mg. The average depression in oxytetracycline (OTC) urine levels produced by TA in these trials was 35 percent as compared with no TA. The magnitude of this depression becomes even more striking when one considers that, in the presence of terephthalic acid, OTC blood serum levels averaged 43 percent greater than in its absence. It can be concluded that the depression in urine antibiotic level produced by TA does not result from diuresis.

TABLE I

The influence of terephthalic acid on urine output in chickens

Trial no.	Terephthalic acid dose (mg)	Average 0-4 hour urine output (ml)	
		OTC* only	OTC* + terephthalic acid
21	132.8	9.55	9.78
24	66.4	7.18	6.85
25	66.4	5.84	6.39
26	66.4	5.50	7.92
27	66.4	6.26	5.32
Average		6.87	7.25

* 4 mg dose

The behavior of OTC serum, liver and urine levels when TA and glucosamine HCl or glucosamine alone was administered with antibiotic into the duodenal loop, as determined in experiment 2, is shown in table 2. Glucosamine has been found to consistently increase OTC serum levels in chickens. The simultaneous increase in urine levels which was obtained indicates that this compound affects absorption of antibiotic rather than its renal excretion.[6]

In the present study, TA produced increases in serum and liver concentrations of OTC in the absence of exogenous calcium which were statistically significant. Glucosamine also increased serum and liver levels under the same conditions. Antibiotic urine levels after glucosamine treament approximated those of the control, whereas urine levels after TA were significantly depressed. The average OTC serum level of birds receiving both glucosamine and TA was higher than with any other treatment. Urine levels also were depressed by the combined treatment.

TABLE II

Effect of 0.4 millimole terephthalic acid, glucosamine, and the combination on serum, liver urine levels of oxytetracycline in the presence and absence of 0.4 millimole exogenous calcium

Treatment	Average OTC concentration (μg/ml or gm) Serum a	Liver b	urine a
OTC, 4 mg	0.41	0.95	120.5
+ TA, 66.4 mg	0.55*	1.32*	65.8†
+ Glucosamine, 71.7 mg	0.47	1.18	113.9
+ TA + Glucosamine	0.58*	1.25	68.7†
OTC, 4 mg + Ca, 16 mg	0.22†	0.62	55.1†
+ TA	0.34#	0.78	23.1†
+ Glucosamine	0.32#	0.83	76.9†
+ TA + Glucosaminec	0.44#	1.70*	58.1†

a = Average of 2- and 4-hour specimens.
b = 4-hour specimens.
* Significantly higher value than OTC.
\# Significantly higher value than OTC + Ca.
† Significantly lower value than OTC.
c Values for serum and liver OTC significantly higher than with TA or glucosamine alone in presence of exogenous Ca.

Exogenous calcium, simultaneously administered with antibiotic, lowered serum, urine, and liver OTC concentrations, only the latter failing to be significantly depressed. Addition of glucosamine, TA or the combination caused significant increases in serum levels in the presence of exogenous calcium. The concentration of antibiotic in the serum and liver of birds receiving both compounds was also found to be significantly higher than with either compound alone. Quantitatively, the responses to the combination appeared to be additive. Although urine levels in all groups receiving Ca were significantly lower than in those not given this cation, glucosamine showed some ability to reverse the depression. Terephthalic acid showed a tendency (non-significant) to reduce further urine concentrations already markedly suppressed by calcium.

The additivity demonstrated in this experiment is strong evidence that glucosamine and terephthalic are complementary in their antibiotic blood level enhancing activities. The data also suggest that the mode of action of the two compounds is different. It is possible that glucosamine increases absorption of OTC and that TA then tends to maintain higher serum levels by slowing the elimination rate of the antibiotic.

Terephthalic acid and Na terephthalate given by the subcutaneous (SQ) route significantly increased serum levels of IM administered OTC, as shown by data of experiment 3 in table 3. A corresponding rise in liver level was also obtained, statistically significant in the case of the salt. Na terephthalate, given IM or IV, failed to increase serum levels although significantly enhancing the antibiotic concentrations in the liver. All TA or Na terephthalate treatments significantly depressed urine levels of antibiotic. The systemic effect exerted by Na terephthalate in this experiment indicates strongly that this drug influences renal excretion of OTC, rather than intestinal absorption. In view of the severely depressed urine concentrations and extremely high liver levels obtained after IM or IV injection, the response of serum levels to these routes of administration seemed unaccountably small.

TABLE III

The influence of terephthalic acid given by various routes on the serum, liver and urine levels of intramuscularly administered OTC

Treatment	Route of administration	Average OTC concentration (μg/ml or gm) Serum a	Liver b	urine a
4 mg OTC	(IM)	1.11	2.68	214.1
+ 66.4 mg terephthalic acid	(SQ)	1.41*	3.49	96.1†
+ 84.0 mg Na terephthalate	(SQ)	1.52*	4.06*	142.6†
+ 84.0 mg Na terephthalate	(IM)	1.18	4.64*	64.4†
+ 84.0 mg Na terephthalate	(IV)	1.21	4.19*	46.9†

a = Average of 2 and 4 hour specimens.
b = 4 hour specimens.
* Significantly higher value than OTC.
† Significantly lower value than OTC.

An additional experiment (4) was conducted to study the effect of 168 mg SQ Na terephthalate on antibiotic specimen levels following IM administration of 4 mg OTC. Results are shown in figure 1.

In the birds given sodium terepthalate, serum OTC contrations were higher throughout the period of 2 to 6 hours over which samples were taken. Also, the absolute difference between serum levels in these two groups was almost the same over the time range studied. This suggests that the maximum excretion-retarding effect of sodium terephthalate occurred

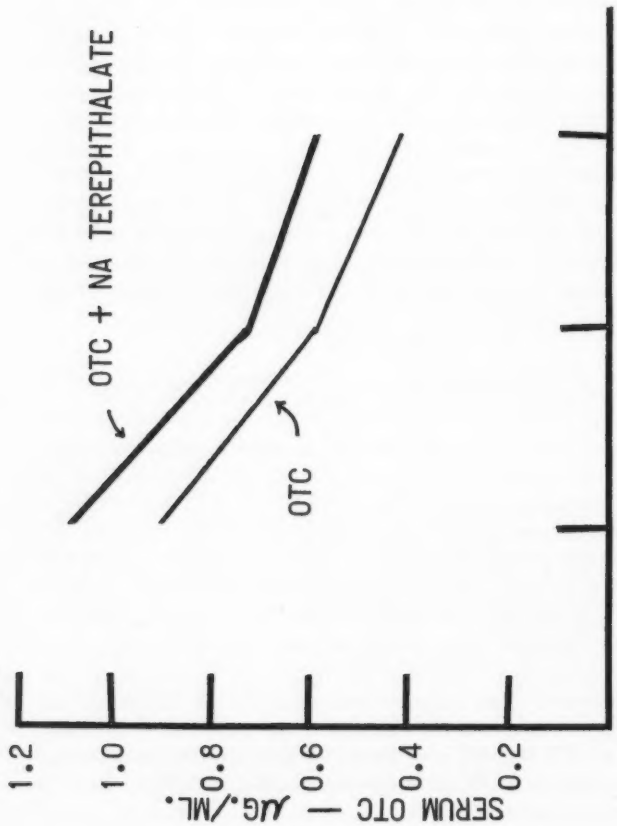

Fig. 1. Response of OTC serum levels to subcutaneously administered sodium terephthalate.

during the first two hours after injection. When level or log level serum OTC was plotted against time, marked parallelism was noted between the two groups. If an excretion-retarding effect had continued, plots would not have been parallel but the OTC serum levels of the Na terephthalate-treated group would have progressively increased over those of the control. This finding could be interpreted to mean that TA or its salt is rapidly eliminated from the body so that a single dose exerts its retarding effect upon excretion for only a short time. In this connection, Porcher[4], working with dogs, found that 75 percent of TA administered in small oral doses is excreted unchanged in the urine. He suggested that the remaining 25 percent was metabolized by the animal, since none was recovered from the feces. He further indicated that, in contrast to most carboxylated aromatic compounds, TA did not conjugate with glycine. Although similar studies have not been conducted in birds, there would appear to be at least one area of physiological activity of TA in this species which differs from that of mammals where it has been shown that TA has somewhat less ability to increase OTC serum levels.[6]

The finding in the present study that the period of activity of Na terephthalate may be limited could be of considerable importance in studies directed toward determining how the acid increases serum OTC levels in birds. Moreover, such information has an important bearing on application of TA as an adjuvant in antibiotic therapy. It indicates that feed use of TA would be rational, as it assures an intake over a period of time coincident with the intake of antibiotic.

It is possible that the key to the action of TA may lie in the differences that exist between avian kidneys and those of mammals. An important difference is that the tubules are a much more important route of secretion or excretion in birds than in mammals.[8] It is therefore possible that a drug which suppresses tubular secretion of an antibiotic may be much more effective in birds than in mammals. Unfortunately, no data are available to show the renal excretion mechanism of the tetracycline antibiotics in birds. However, it has been demonstrated by Engelund[3] that in rabbits approximately 50

percent of tetracycline elimination takes place through tubular secretion. Contrasting results in humans were reported for chlortetracycline* by Sirota and Saltzman[7] who showed that the renal excretion of this antibiotic is solely a process of glomerular filtration. This indication of possible pharmacological difference between the tetracycline antibiotics or variation in species response makes further study on the route of elimination of OTC in birds imperative.

In addition, in birds, ornithine rather than glycine is the principal detoxifier of aromatic acids such as benzoic.[1] The conjugation of benzoic acid and ornithine results in the formation of dibenzoylornithine, or ornithuric acid. If ornithine plays a role in the elimination of OTC, and TA preferentially conjugates with this amino acid, the antibiotic may be less subject to glomerular filtration or tubular secretion, thus maintaining antibiotic at a higher concentration in the circulating blood.

Figure 2 shows comparative OTC serum values obtained in experiment 5 in which Na terephthalate was injected SQ immediately after duodenal loop blood vessels had been ligated to stop further absorption of antibiotic administered one hour previously. Increased serum levels obtained under these conditions would indicate, of course, that TA could potentiate without necessarily increasing absorption of antibiotic. Although the differences between blood serum values of birds receiving OTC and Na terephthalate and those that received OTC only did not attain statistical significance, there is some indication that the salt helped to raise antibiotic serum concentrations to a higher level, despite the fact that, at zero time, the average blood level for the Na terephthalate group was somewhat lower than the control. A fairly constant increase in the serum OTC levels was found after the 1-hour sampling, again indicating that the salt exerted its effect shortly after administration.

Results of experiment 6, in table 4, provide a comparison of blood level enhancing effects of TA and of its

* The trade mark of Lederle Laboratories Division, American Cyanamid Co. for chlortetracycline is Aureomycin.

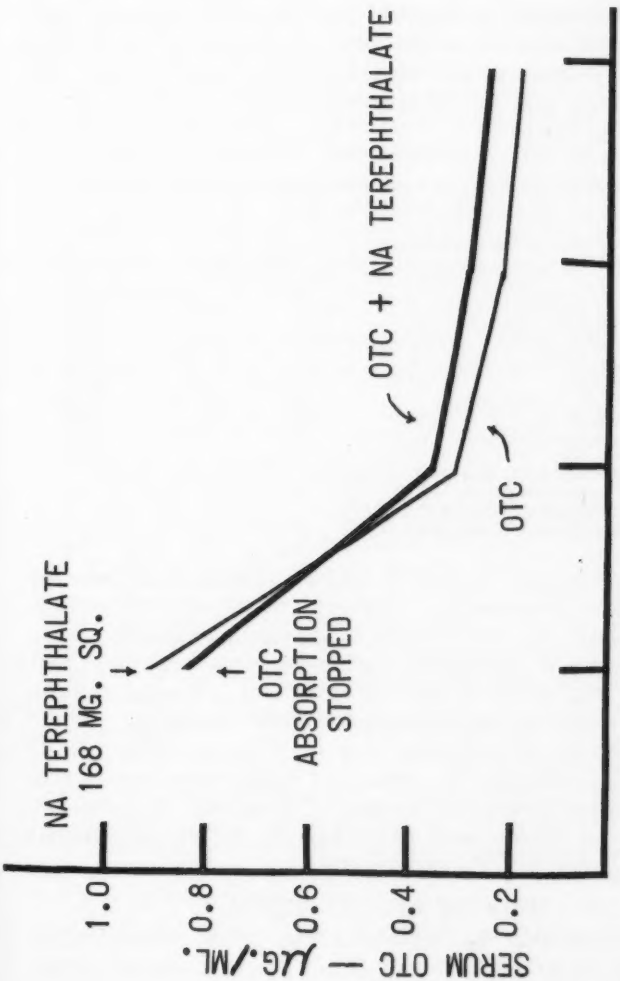

Fig. 2. Effect of Na terephthalate on OTC serum levels when given after further absorption of antibiotic (administered to the duodenal loop 1 hour previously) was prevented.

structural isomers and certain related compounds. Among this group of compounds, only TA showed ability to increase significantly OTC serum and liver levels. The marked increase in specimen antibiotic concentrations obtained was accompanied by an atypically small depression of urine OTC level. The isomers of this acid, isophthalic and phthalic, did not detectably affect OTC specimen levels.

TABLE IV

The effect of isomers and analogs of terephthalic acid on blood serum, liver and urine levels of OTC

Treatment	Dose, mg (equivalent to 0.4 millimole)	Average OTC concentration (μg/ml or gm) serum a	Liver b	urine a
OTC, 4 mg	—	0.45	0.94	159.0
+ Terephthalic acid	66.4	0.84*	1.70*	125.8
+ Isophthalic acid	66.4	0.41	0.86	140.5
+ Phthalic acid	66.4	0.42	1.31	147.4
+ Benzoic acid	48.8	0.50	1.00	107.5†
+ Dimethyl terephthalate	79.2	0.47	1.28	99.8†
+ Probenecid	114.1	0.30†	0.87	38.5†
+ P-hydroxybenzoic acid	55.3	0.28†	1.04	60.0†

a = Average of 2 and 4 hour specimens.
b = 4 hour specimens.
* Significantly higher than OTC.
† Significantly lower than OTC.

The other four compounds tested all significantly lowered urine OTC concentrations. However, no appreciable change in serum level was produced by benzoic acid or dimethyl terephthalate, while probenicid and p-hydroxbenzoic acid significantly depressed serum concentrations. No explanation for these apparent anomalies is presently available.

The above data suggest that the chemical structure of compounds producing the enhancing effect described in this series of experiments is quite specific since only the p-carboxy derivative of benzoic acid (TA) and its sodium salt showed ability to enhance OTC serum levels.

SUMMARY AND CONCLUSIONS

1. The depression in oxytetracycline urine concentration caused by terephthalic acid is not due to a diuretic effect but to a reduction in the elimination rate of the antibiotic.
2. Simultaneous administration of glucosamine, a drug which

appears to increase OTC absorption from the intestine, and terephthalic acid produced an increase in OTC serum levels which appeared to be additive in nature.

3. The subcutaneous administration of terephthalic acid or its sodium salt concurrently with intramuscular injection of OTC resulted in significantly increased antibiotic serum levels and depressed urine levels.
4. Terephthalic acid, given by the same route, but after intestinal absorption of antibiotic had been stopped, showed consistent, although non-significant, ability to maintain serum levels of OTC above those of controls.
5. Neither the isomers of terephthalic acid, isophthalic and phthalic acids, nor several structurally related compounds significantly increased OTC serum levels.

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THE SUSCEPTIBILITY OF A TURKEY ORNITHOSIS VIRUS OF LOW VIRULENCE TO ANTIBIOTICS

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THE chicken embryo is highly susceptible to infection by viruses of the psittacosis-lymphogranuloma group and has been used by numerous workers in studying these viruses.[1,7,9,10] The embryo technique also provides an accurate method for studying the camparative value of different therapeutic agents.[2,5] The purpose of this paper is to report the effect of several antibiotics against a psittacosis-lymphogranuloma virus of low virulence and toxicity for turkeys and mice.[4]

MATERIALS AND METHODS

An egg-propagated strain of this turkey virus in yolk sac material served as the inoculum. The virus had been recovered from turkey tissues being screened for the infectious sinusitis agent*. The embryonated eggs were obtained from a flock of White Leghorn chickens fed a balanced ration free of antibiotics. Six-day old chicken embryos were inoculated via the yolk sac with 0.2 ml of the virus serially diluted in a phosphate buffer from 10^{-1} through 10^{-8}.

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* Appreciation is expressed to Dr. B. S. Pomeroy of the University of Minnesota for furnishing the virus for this study.

Data used in this paper were submitted to the Graduate School of the University of Minnesota in partial fulfillment for the degree of Doctor of Philosophy.

The eggs were inoculated in the yolk sac with 0.1 ml of virus and thirty minutes later the antibiotic was introduced. Eight antibiotics at the following levels per egg were used: streptomycin 1, 1.25, 25 and 50 mg, dihydrostreptomycin 1.25 and 2.5 mg, neomycin 1 and 2 mg, magnamycin 1 and 2 mg, penicillin 100, 1000 and 5000 units, chlortetracycline 1 and 2 mg, oxytetracycline 1 and 0.5 mg and erythromycin 0.5 mg. Chicken embryo controls included uninoculated embryos, virus inoculated, phosphate buffer inoculated (diluent) antibiotics and buffer in the same dosage as cited above. Two or more trials were carried out for each antibiotic examined, with 12 or more eggs for each trial. Embryo deaths occurring from the third through the tenth day were used for determining the endpoint. The titration endpoint was determined by the Reed and Muench method.[11] The yolk sac material was plated on blood agar, PPLO agar, and inoculated into thioglycollate media for sterility checks. Yolk sac membranes were stained by Macchiavello's method to determine the presence of elementary bodies.

RESULTS

The LD_{50} titer of the virus was found to average $10^{-5.85}$, for five consecutive passages. The greatests percent mortality occurred between the fourth and sixth day. The virus has been passed for over 65 passages in chicken embryos with no apparent loss in pathogenicity. The embryos were hemorrhagic and stunted (figure 1). The yolk sac often appeared watery and thin. Macchiavello stained smears from the yolk sac revealed the presence of elementary bodies. These findings are similar to those found with highly virulent strains of virus on embryo passage.

Oxytetracycline and erythromycin were found to be the most effective antibiotics in prevention of chicken embryo mortality (table 1). They furnished 100 percent protection to the inoculated chicken embryos. Streptomycin, dihydrostreptomycin, neomycin and magnamycin were of no apparent value in these trials, permitting 100 percent mortality in all but one instance. Chlortetracycline was effective in preventing mortalities but required higher levels than either oxytetra-

cycline or erythromycin. Penicillin was not as effective as the broad spectrum antibiotics. It did, however, at high levels, prevent mortality. At the low level of 100 units per egg, the protection offered was slightly better than 41 percent, while at the higher levels, it approached in effectiveness that of the broad spectrum antibiotics.

TABLE I

Susceptibility of the virus to antibiotics

Antibiotic	*Dose Level per egg*	*Chicken** embryos*	*Percent* mortality*
(Virus control)	None	24/0	100
Streptomycin	1.00 mg	24/0	100
Streptomycin	1.25 mg	24/0	100
Streptomycin	25.00 mg	24/0	100
Streptomycin	50.00 mg	24/0	100
Dihydrostreptomycin	1.25 mg	24/0	100
	25.00 mg	24/0	100
Neomycin	1.00 mg	24/0	100
Neomycin	2.00 mg	24/0	100
Magnamycin	1.00 mg	24/0	100
Magnamycin	2.00 mg	20/4	83
Penicillin	100 units	14/10	58
Penicillin	1000 units	8/12	40
Penicillin	5000 units	3/21	12
Chlortetracycline	1 mg	4/20	17
	2 mg	1/23	4
Oxytetracycline	1 mg	0/24	0
Oxytetracycline	0.5 mg	0/24	0
Erythromycin	0.5 mg	0/24	0

* 10 day period of observation
** Numerator indicates number of chicken embryos which died. Denominator indicates number that survived.

DISCUSSION

The virus was found to fall into the antibiotic susceptibility pattern established for the psittacosis lymphogranuloma group of viruses by previous workers.[2,3,5,8] It is interesting to note that although this virus in turkeys was of very low virulence, the chicken embryo responded with the same sensitivity to this strain as to the virulent strains[2].

The treatment of avirulent strains may seem unimportant since such strains produce only little or no mortality. While this may be true, it nevertheless becomes important to eliminate such infections if compete control of the disease is to be

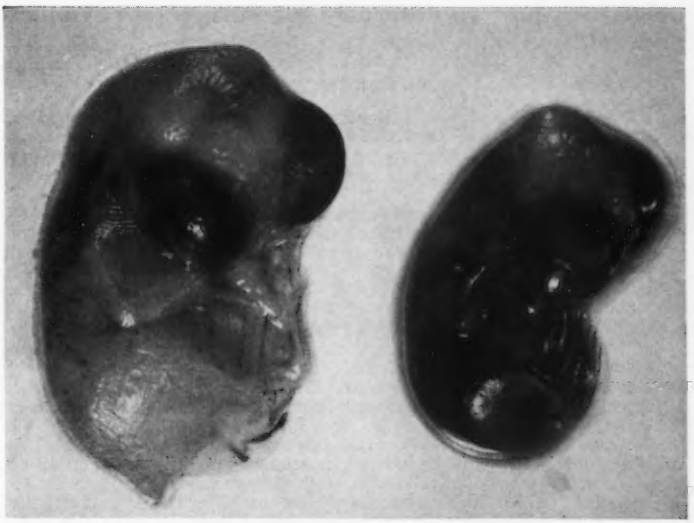

Fig. 1. Chick embryos eleven days of age. The embryo at the left is a control. The embryo at the right died five days after inoculation with 0.2 ml of the virus through the yolk sac. Note the smaller size and hemorrhagic appearance .

realized. One further point to consider is that the virulence is one of degree and that human infections with this virus have been reported.[6] In addition to these factors, the resemblance of lesions from this infection to respiratory lesions of other infections may complicate the diagnosis of other diseases, especially chronic respiratory disease or infectious sinusitis. This is especially true if mixed infections are encountered.

SUMMARY

A virus of the psittacosis lymphogranuloma group of low virulence for turkeys was tested for susceptibility to antibiotics in chicken embryos. Oxytetracycline and erythromycin were effective in preventing 100 percent embryo mortality while streptomycin, dihydrostreptomycin, neomycin and magnamycin were not. Chlortetracycline required higher levels than oxytetracycle or erythromycin to be effective. Penicillin

at high levels prevented mortality but not as effectively as the broad spectrum antibiotics.

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STUDIES ON AVIAN BACTERIAL L-FORMS
I. A METHOD OF IDENTIFICATION

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THE demonstration by Dienes[4] of the relationship between the L-1 organism of Klieneberger[7] and *Streptobacillus moniliformis* opened a new area in the field of bacteriology. Many other bacterial species have been shown to undergo transition into the L-form[5] but the complete significance of this phenomenon has not been determined. The morphology, growth requirements and general characteristics of bacterial L-forms are the same as has been given for the organism *Mycoplasma*[2], which are more commonly known as PPLO. The only significant difference is that the L-form will, under certain conditions, revert to the bacteria from which they originated. Therefore, all organisms having the characteristics of PPLO must be considered as members of that group until they are proven otherwise by reversion to their bacterial form. An understanding of the nature of L-forms and their relationship to avian PPLO is important in studies on chronic respiratory disease (CRD) of chickens. PPLO are associated with the etiology CRD, but only certain strains are capable of producing infection.[1] Some of the organisms may actually be L-forms and a method for their identification is needed.

Various techniques have been used to obtain bacteria from L-forms[4] but no simple method has been found which

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will give consistent results.[3,6] When bacteria appear in an L-form culture, the source of these bacteria must be determined. No techniques can be considered absolute in the prevention of contamination and the bacteria must be identified either as having reverted from the L-form or as contaminants.

Cultures designated as avian PPLO were obtained from various research laboratories. These were carried through serial passage in broth and when bacteria were observed, the question of whether they were a result of reversion or contamination had to be considered. A group of bacterial cultures which were considered to have reverted from L-forms was selected. This selection was based on the frequency of the appearance of bacteria of the same type from a large number of cultures of a given PPLO strain. The bacteria recovered were routinely gram positive cocci, apparently micrococci.

The reverted bacteria consistently appeared as pure, bacterial colonies without L-colonies on the plates containing no inhibitors, and as pure L-cultures on the plates containing inhibitors. Bacteria which had been isolated as contaminants by the intentional exposure of plates in the laboratory grew readily as bacteria on the plates containing no inhibitors, and never produced L type colonies on the inhibitor agar.

Using the type of growth on agar as a basis for comparison, it was considered possible to determine the identity of a bacterium as either a reversion from an L-form or as a contaminant by the use of agar plates as shown in table 1. L-form cultures would produce L-colonies on both types of media while bacteria reverted from an L-form would produce L-colonies on inhibitor agar and bacterial colonies on the non-inhibitor agar. A contaminant, although growing as bacteria on the non-inhibitor agar the same as reverted bacteria, would show no growth on the inhibitor agar or in some cases, overcome the small amount of inhibitors employed and grow as bacteria. A contaminant and L-form mixture would produce both L-form and bacterial colonies on non-inhibitor agar and either both or L-form colonies alone on inhibitor agar. This last mixture of L-form and contaminant is the type which must be detected and not confused with bacteria which have re-

verted from an L-form The purpose of this paper is to outline a procedure for such a differentiation.

MATERIALS AND METHODS

To determine if the assumptions outlined in table 1 were valid, cultures of the various types of organisms were prepared. Five L-cultures and their reverted bacteria were selected on the basis of: (a) the frequency of reversion in broth, (b) the uniformity in the type of bacteria obtained from the L cultures, and (c) their growth on inhibitor and non-inhibitor agar. Five contaminants were selected from agar plates which had been exposed in the laboratory. Specific identification of these contaminants was not attempted but they included two cultures of gram positive cocci, a gram negative rod, a gram positive rod and a gram positive diplococcus.

TABLE I

Type of colonies expected with various inoculums on inhibitor and non-inhibitor agar

Inoculum	*Type of colonies*	
	Inhibitor agar	*Non-inhibitor agar*
L-form	L*	L
Bacteria reverted from L-form	L	B**
Bacterial contaminant	B or Neg.	B
L-form and bacterial contaminant	L or L and B	L and B

* L—L-form colonies
** B—Bacterial colonies

The media used in all tests were Difco beef heart infusion agar and broth to which were added 1 percent Difco proteose peptone #3 and 1 percent Difco yeast extract. Sterilization was carried out at 121 C for 15 minutes. The media were cooled to at least 50 C before adding 1½ percent of iDfco PPLO serum fraction. When inhibitors were desired, penicillin and thallium acetate were added in amounts sufficient to give 500 units per ml and a dilution of 1:4000 respectively. These were added at the same time as the serum fraction. All broth tubes and agar plates were incubated 48 hours before use and checked for evidence of contamination.

Broth cultures were prepared for the individual organisms (L-forms, reverted bacteria and contaminants) and after 24 hours incubation 0.1 ml was plated on inhibitor and non-inhibitor agar. The mixtures of L-forms and contaminants were prepared by adding 0.1 ml of contaminant culture to 2.5 ml of L-culture. These mixed cultures were then inoculated onto both types of agar after 3, 24, and 96 hours incubation at 37 C. All plates were examined grossly for bacterial colonies and microscopically at 100 magnification for L-colonies after 24, 48, and 96 hours incubation. There was no difference in the findings at these various times so all results are based on the 96 hour readings.

RESULTS

The cultures of L-forms, reverted bacteria, and contaminants produced the kind of colonies that had been expected (table 1) when plated on inhibitor and non-inhibitor agar. The L-form cultures gave L-colonies on both types of agar while the reverted bacteria gave L-colonies on inhibitor agar and bacterial colonies on the non-inhibitor agar. All five cultures of the contaminants grew as bacteria on non-inhibitor agar and two gave a few bacterial colonies on the inhibitor media. No L-form type colonies were found.

The type of colonies obtained from the mixtures of L-forms and contaminants varied. When plated after 3 hours incubation, the results were similar to those in table 1 for a reverted bacterium. As is shown in table 2, results of the same type were obtained when they were plated after 24 hours incubation. The L-forms had apparently remained viable for at least 24 hours as indicated by their growth on the inhibitor agar. However, when plated after 96 hours incubation the L-forms had been eliminated and only the bacterial contaminants grew.

When attempting to revert L-forms to bacteria, the cultures are plated as soon as bacterial turbidity is observed. From the results shown in table 2 it can be seen that the presence of a bacterial contaminant in an L-culture would give results similar to those expected for a reverted bacterium unless the culture was allowed to incubate at least 96 hours.

If plated before that time identification of the bacterium as either a reversion or a contaminant could not be made. This difficulty was overcome by subculturing the bacteria which grew on the non-inhibitor plate. A well isolated single bacterial colony was picked and inoculated into broth. After 24 hours incubation, the resulting culture was plated on inhibitor and non-inhibitor agar as had been done with the original culture. A bacterium which had reverted from an L-form always produced L-colonies on inhibitor agar and bacteria on non-inhibitor plates. The contaminants failed to produce L-forms and were either negative or grew as bacteria on the inhibitor agar. The use of broth for this subculture was necessary since the reverted bacteria apparently became stabilized in the bacterial form when transferred directly from one agar plate to another.

TABLE II

Growth obtained with various inoculums on inhibitor and non-inhibitor agar

Inoculum	Type of colonies			
	Inhibitor agar		Non-inhibitor agar	
	L*	B**	L	B
L-forms	5/5	0/5	5/5	0/5
Bacteria reverted from L-form	5/5	0/5	0/5	5/5
Bacterial contaminant	0/5	2/5	0/5	5/5
L-form and bacterial contaminant—3 hr. incub.	25/25	8/25	4/25	25/25
L-form and bacterial contaminant—24 hr. incub.	24/25	10/25	0/25	25/25
L-form and bacterial contaminant—96 hr. incub.	0/25	10/25	0/25	23/25

* L—L-form colonies
** B—Bacterial colonies

A procedure for the identification of a bacterial culture as a reverted L-form is shown in figure 1. When bacterial growth is observed in an L-form culture it is plated on inhibitor and non-inhibitor agar (A-figure 1). The plates are examined daily and if no L-colonies are found on the inhibitor

agar after 96 hours incubation, the bacterium is classified as a contaminant. When L-colonies are observed, a single isolated bacterial colony is then picked from the non-inhibitor plate into broth (B-figure 1). After 24 hours incubation this culture is transferred to both types of agar. The presence of L-colonies on the inhibitor agar and bacterial colonies on the non-inhibitor agar identifies the original bacterium as a reversion from the L-form.

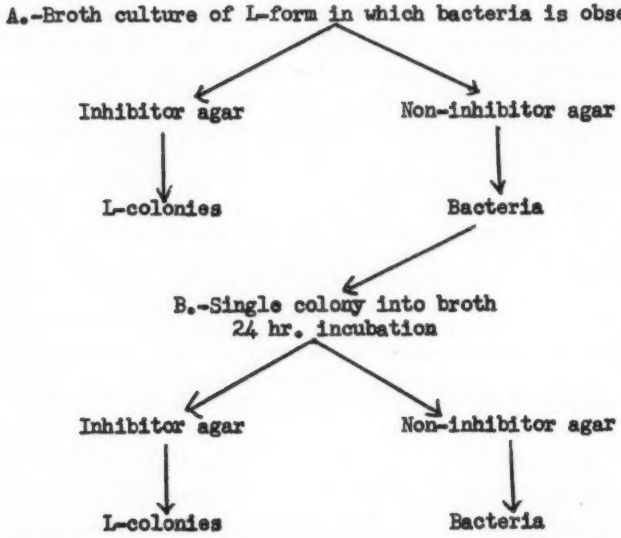

Fig. 1. Procedure for the identification of bacterial culture as a reverted L-form.

SUMMARY

Bacteria were frequently recovered from cultures of avian PPLO. The source of such bacteria was either reversion or contamination. If reversion had taken place, the original culture was considered a bacterial L-form. A method is outlined for the identification of bacterial L-forms by their cultivation on inhibitor and non-inhibitor agar. The growth ob-

tained with mixtures of L-forms and contaminants was often similar to the growth pattern obtained with reverted bacteria. These could be differentiated by subculturing in broth and additional plating on the selective agars

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THE ISOLATION OF VIRUSES FROM THE CECAL TONSILS OF CHICKENS AND TURKEYS

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IT IS the purpose of this paper to report the isolation of viral agents from the cecal tonsils of chickens and turkeys. Routine virus isolation studies on poultry specimens with respiratory infection presented to this laboratory resulted in isolations from the tracheal tissue during the early peracute stage of infection. This tissue often yielded no virus from birds during the latter phases of respiratory disease. At necropsy, those birds with persistent respiratory distress, had enlarged and inflamed lymphoid tissue in the proximal area of the ceca, tissue usually referred to as cecal tonsils. Since there seemed to be a relationship between respiratory infections and the gross lesions in this tissue, we theorized that the change may have been due to invasion by virus. Accordingly this tissue was selected for virus isolation studies.

MATERIALS AND METHODS

The cecal tonsil in the proximal area of each cecum was dissected from adjoining tissue and rinsed free of cecal contents in saline. The selective fluorocarbon deproteinization with Genetron 113* (trifluorotrichloroethane) as outlined by Gessler *et al.*[2] was used. The cecal tissue (approximately 2 gm)

* Allied Chemical and Dye Corp., New York City.

was ground in a mortar with sand and made into a suspension with 10 ml McIlvaine citrate buffer pH 7.4, diluted 1:100 in physiological saline, plus 5 ml of Genetron. The preparation was put into a sterile pyrex rubber stoppered test tube and gently agitated to keep the mixture uniformly mixed for 5 minutes. The preparation was centrifuged for 5 to 10 minutes at 1,000 x g., the supernatant, aqueous phase removed and treated with 4,000 units of penicillin and 30 mgs of dihydrostreptomycin per milliliter for one hour at room temperature.

The treated suspension (0.2 ml) was inoculated onto the chorioallantoic membrane of ten-day-old chicken embryos. Eggs were candled daily and any deaths occurring during the first 24 hours of incubation were considered to be due to trauma; however, a hemagglutination test for Newcastle disease virus was always made. Embryos dying after 24 hours were stored in the refrigerator. At the end of the seven day incubation period, both dead and live embryos were examined individually. Membranes showing pock-like lesions, edematous areas, cloudy areas, or cysts or blebs, were selected for harvesting. Only the area showing lesions or in the case of a blind passage, the inoculated area in the false air cell, was dissected free of adjoining tissue and used as seed for the next passage. The harvested tissue was ground and treated with Genetron in the same manner as the original tissue for repassage. Usually three blind passages were made before an attempt was considered negative.

Most of the isolates produced lesions on the chorioallantoic membrane or embryo death on the first passage. Some required several passages to become embryo adapted. Once an agent was established and apparently stabilized, it was passed without Genetron treatment. Bacteriological sterility checks were made on each passage. Isolations from other tissues were done in the same way as described for cecal tonsils.

Tentative identification of isolates was done on embryo pathology produced. Further identification was done, where possible, by serological tests. Hemagglutination positive iso-

lates were tested by the hemagglutination inhibition test for Newcastle disease virus. In some instances, the strain of Newcastle disease virus was further characterized by chick inoculation. Serum neutralization tests with immune sera for laryngotracheitis virus were done on all agents not otherwise classified. Macroscopic lesions produced on the chorioallantoic membrane were also studied by histopathological techniques which enabled further differentiation of these isolates.

RESULTS

Cecal tonsils were collected for attempted virus isolation from 88 cases of respiratory disease. Forty-four separate isolations were successful from these cases. In 74 of these 88 cases, both the cecal tonsils and tracheas were processed for virus isolations. Virus isolations were made from 39 of these cases, with 36 derived from the cecal tonsils and 23 from the trachea. Virus isolations were successful from both the cecal tonsils and trachea of 20 cases. In 16 cases the cecal tonsils were positive and the trachea negative; in three cases the cecal tonsils were negative and the trachea positive.

Representative cases illustrating the results obtained are summarized in table 1. The cases are classified in groups according to symptomatology as A, peracute; B, acute; C, persistent; D, no visible pathology but with lowered feed conversion and a low carcass grade; E, miscellaneous signs other than respiratory. Five turkey cases are included in the table.

It can be seen from table 1 that in the peracute and acute stages of respiratory disease, isolations were generally successful from both the trachea and cecal tonsils. In the persistent cases and those with no visible pathology, the tracheas were negative but isolations were made from the cecal tonsils.

DISCUSSION

In peracute and acute disease, the isolation of viruses should be a relatively simple matter since in such cases virus is usually present in excess. However, in chronic cases and those in which no visible pathology exists, the viral agent must be in a kind of equilibrium system with inhibitors which tend to check its multiplication and spread. An obvious in-

Table I

Summary of Virus Isolations

No.	Age	Tissue	Results
Group A (Peracute Respiratory Distress)			
74	13 wks.	Trachea	LT
		C.T.	Uncl.
121	4 wks.	Trachea	NDV
		C.T.	NDV
76	4 wks.	Trachea	NDV
		C.T.	NDV
91	3 wks.	Trachea	Bron.
		Liver	Neg.
		C.T.	Neg.
Group B (Acute Respiratory Distress)			
64	6 wks.	Trachea	Uncl.
		C.T.	Uncl.
65	6 wks.	Trachea	LT
		C.T.	LT
93	Adult	Trachea	NDV
		C.T.	Uncl.
39	9 wks.	Conj.	NDV
		Trachea	Neg.
		C.T.	NDV
56	6 wks.	Trachea	Fowl pox
		C.T.	Uncl.
58	6 wks.	Eye	NDV
		Trachea	NDV
		C.T.	NDV
67	6 wks.	Eye	NDV
		C.T.	NDV
Group C (Persistent Respiratory Distress)			
50	12 wks.	Brain	NDV
		Spinal cord	NDV
		Trachea	Neg.
		C.T.	Uncl.
78	12 wks.	Trachea	NDV
		C.T.	NDV
125	10 wks.	Trachea	Neg.
		C.T.	Fowl pox
		Bur. Fab.	Neg.
9	4 mo.	Trachea	Neg.
		C.T.	Uncl.
23	6 wks.	Trachea	Neg.
		C.T.	Fowl pox
Group C (Cont'd.)			
97	9 wks.	Trachea	Neg.
		Spleen	NDV
		C.T.	Uncl.
145	7 wks.	Trachea	Neg.
		C.T.	Uncl.
68	8 wks.	Trachea	Neg.
		C.T.	Uncl.
55	4 wks.	Eye	Neg.
		Trachea	Neg.
		Brain	Neg.
		C.T.	NDV
82	12 wks.	Trachea	Neg.
		C.T.	NDV
Group D (No Visible Pathology)			
150	10 wks.	Brain	Neg.
		Trachea	Neg.
		C.T.	Uncl.
151	4 wks.	Brain	Neg.
		Trachea	Neg.
		C.T.	Fowl pox
152	10 wks.	Brain	Neg.
		Trachea	Neg.
		C.T.	Uncl.
Group E (Miscellaneous)			
43	16 wks.	Brain	Neg.
		C.T.	Uncl.
84	Adult	Wattles	Neg.
		C.T.	Uncl.
59	Adult	C.T.	NDV
66	8 wks.	Eye	Neg.
		C.T.	NDV
48	4 wks.	Eye Humor	Neg.
		Hard. gl.	Uncl.
		C.T.	Uncl.
Turkey			
135		C.T.	Uncl.
49	10 days	C.T.	Uncl.
78	2-3 mo.	Brain	Neg.
		Trachea	Neg.
		C.T.	Uncl.
102	3 mo.	C.T.	Uncl.
113	3 mo.	C.T.	Uncl.

C. T. = cecal tonsils; conj. = conjunctiva; Bur. Fab. = bursa of Fabricius; Har. gl. = Harder's gland; LT = laryngotracheitis virus; NDV = Newcastle disease virus; Bron. = infectious bronchitis virus; Uncl. = unclassified; Neg. = negative

hibitor in the animal body is specific antibody which can also be an important interfering factor in virus isolation from such cases. Specific antibody can be removed by treatment with fluorocarbon as was shown by Hummeler and Ketler[5] who were able to free 65 to 70 percent of specifically neutralized type 1 poliomyelitis virus by such treatment.

Proteins, lipoproteins and lipids are removed from the aqueous phase by fluorocarbon. This property was used by Gessler *et al.*[2] and others[1,6] to purify viral suspensions and by Halonen, Huebner and Turner[3] to remove host antigens from ECHO virus complement-fixing antigens. Hamparian, Muller and Hummeler[4] used fluorocarbon to remove anticomplementary activity and other undesirable reactivities from complement-fixing antigens.

The success of the present viral isolations, particularly from birds with persistent infections, may be due largely to the removal of specific antibody from the initial preparations by fluorocarbon, an "unmasking" action. The removal of other interfering proteinaceous materials may be another important factor in successful isolation, particularly since the number of infective virus particles in such preparations is probably small. This has the effect of concentrating the virus in relation to the total protein in the preparations.

The fluorocarbon treatment, although materially aiding in isolating some viruses, may limit the method by inactivating or precipitating other viruses. For example, Hamparian, Muller and Hummeler[4] reported that mumps and influenza viruses were more readily precipitated by fluorocarbon than viruses of the Coxsackie and poliomyelitis groups.

The number of blind passages employed affects the chances of success in obtaining an isolate. Although the number of blind passages in this work was limited to three for practical reasons, additional passages were made in a few instances. The results indicated that an increased number of passages would have resulted in a higher percentage of isolations.

During the acute stages of respiratory disease, the isolation of the causative viral agents may be expected not only from the trachea but from other organs as well because of the high rate of virus production and the relative lack of specific

antibody. However, in the persistent stage, the tissue yielding virus probably represents a depot where the agent may persist for extended periods. Isolations of Newcastle disease virus in cases 97, 55 and 82 are illustrative. These results indicate that the cecal tonsils and possibly other areas of the intestinal tract are such depots where respiratory and probably other viruses can persist. Such depots may aid in explaining the endemicity of what are usually considered acute viral diseases.

SUMMARY

1. A fluorocarbon purification technique was successful for isolating viral agents from cecal tonsils in a high proportion of cases of respiratory disease in poultry.

2. Virus isolations from the cecal tonsils were successful in 44 out of 88 cases. In peracute and acute cases, isolations could be made from both the trachea and cecal tonsils. However, in persistent cases, isolations were usually successful only from the cecal tonsils.

3. Some reasons for the relative success of the fluorocarbon technique are discussed.

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EGG CHARACTERISTICS AS AIDS IN SPECIES IDENTIFICATION AND CONTROL OF CHICKEN TAPEWORMS*

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FEW poultry pathologists are equipped to make specific identification of poultry tapeworms. If species could be determined quickly, the diagnostician could make valuable suggestions to poultrymen in terms of intermediate host control. Wehr[5] has reviewed the extensive life history studies which have been completed on the eight common chicken tapeworms. A careful study of the list of intermediate hosts reveals many possible control suggestions after specific identification has been completed. *Davainea proglottina* calls for slug and snail control. *Raillietina echinobothrida* and *R. tetragona* suggest ant control while *Amoebotaenia sphenoides* is carried by earthworms. *Hymenolepis carioca, H. cantaniana* and *Raillietina cesticillus* are largely beetle problems while *Choanotaenia infundibulum* represents a beetle, housefly or grasshopper problem. Specific identification can assist the diagnostician also in making a flock prognosis since various species range from highly pathogenic to almost harmless parasites.

Specific diagnosis has been delayed in the past by the time consuming methods of staining the specimen. Few laboratories are equipped to complete this identification procedure rapidly enough to be of use to poultrymen. Species identification can be achieved rapidly on fresh specimens

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through comparing the distinctive scoleces with the illustrations of Wehr[5] or Lapage.[2] However, recovery of the scolex in the more fragile specimens is often difficult even for the most experienced technician. Studies in this laboratory indicate that the "eggs" (more properly termed the onchospheres) together with enveloping membranes, are often as distinctive in species determination as the scoleces. This method has the advantage that a specimen may be recovered and identified readily from the larger end of the live worm. If live specimens are examined and compared with the table and illustrations in this paper, species identification can usually be achieved within ten minutes after cestodes are found in the intestine.

MATERIALS AND METHODS

As tapeworms are revealed upon cutting open the intestine, they may be located and isolated more readily if floated out in a shallow dish of water. The intestine should be examined under a low power binocular microscope for smaller specimens. Worms should be isolated in a dish of water. Two or three of the largest proglottids may be cut off and placed on a slide in a drop of water. For examination of eggs, the proglottid should be shredded apart with dissecting needles, covered with a cover glass and examined under the compound microscope. Water mounts of chains of mature proglottids may be prepared for location of the genital pore and the cirrus pouch. Measurements, made with a calibrated ocular micrometer mounted in a compound microscope, will be helpful in distinguishing between species in some genera.

IDENTIFICATION PROCEDURES

Species determination can be made through the use of table 1 and the illustrations. After locating a tapeworm, examine the general shape and length of the strobila. So-called "microscopic forms" (*Amoebotaenia sphenoides* and *Davainea proglottina*) which are less than one-fourth of an inch in length, may be separated from the other six species on the basis of size. If one of these two species is suspected, an examination of the terminal proglottid should be made with the microscope to make certain that the specimen is not an in-

TABLE 1. DISTINGUISHING CHARACTERISTICS IN LIVING SPECIMENS OF THE EIGHT COMMON SPECIES OF CHICKEN TAPEWORMS

Species	Egg Characteristics	Strobila		Proglottid number and shape	Genital Pore Location and Cirrus Characteristics	Scolex Characteristics	Degree of Pathogenicity	Intermediate Hosts
		Length in Inches	Characteristics					
Amoebotaenia sphenoides	single, membranes contain one granular layer	1/4	minute, triangular form	20-30 proglottids, much broader than long	regularly alternate, empty anterior end of lateral margin of proglottid	rostellum armed with single row of 12-14 hooks suckers unarmed	mild	earthworms (4 species)
Davainea proglottina	single, enclosed in membranes formed from uterine wall	1/4	minute forms mostly buried in mucosa	2-9 proglottids, gravid forms longer than broad	alternate regularly, empty very far anterior on lateral margin, voluminous cirrus pouch	rostellum armed with 80-94 minute hooks, suckers small, unarmed	severe	slugs snails
Hymenolepis carioca	single, inner membrane egg-shaped with granular accumulations at poles	3½	thread-like, very fragile never more than 1/8 inch in width	many hundreds much broader than long	unilateral, empty anterior to middle margin of proglottid	rostellum rudimentary unarmed, rostellar sac 30 x 110 μ suckers unarmed	mild or harmless	grain beetles
Hymenolepis cantaniana	single, similar to H. carioca	3/4	thread-like, very fragile	numerous, much broader than long	unilateral, similar to H. carioca	rostellum rudimentary, smaller than H. carioca (sac 30 x 70 μ), suckers unarmed	mild or harmless	dung beetles
Raillietina tetragona	6-12 eggs enclosed in a capsule	9½	large, robust	numerous, broader than long	usually unilateral, opening middle to anterior portion of proglottid. Cirrus pouch small 75-100 u	suckers oval, armed with 8-12 rows of hooks (8-10 μ) rostellum with single or double row of 100 hooks	moderate to severe	ants ?
Raillietina echinobothrida	similar to R. tetragona	9½	similar to R. tetragona	similar to R. tetragona	usually unilateral, opening posterior to middle portion of proglottid. Cirrus pouch larger 130-180 u	suckers round, armed with 8-15 rows of hooks (10-13 μ). Rostellum with double row of 200 hooks. Both kinds of hooks larger than R. tetragona	severe, causes nodular lesions	ants ?
Raillientina cesticillus	single, large, mature specimens show two funnel-shaped filaments on inner membranes	5	large, robust difficult to loosen from the mucosa	numerous, gravid forms of equal length and width	irregularly alternate, empty anterior to middle portion of proglottid	unusually broad, flat rostellum armed with double row of 300-500 hooks, suckers weak, unarmed	moderate	grain beetles ground beetles dung beetles
Choanotaenia infundibulum	2 distinctive elongated filaments on poles of mature eggs	7	large, robust	numerous gravid forms longer than broad	irregularly alternate, empty in anterior third of proglottid	rostellum armed with single row of 16-20 large hooks suckers unarmed	moderate	grain beetles house flies grasshoppers

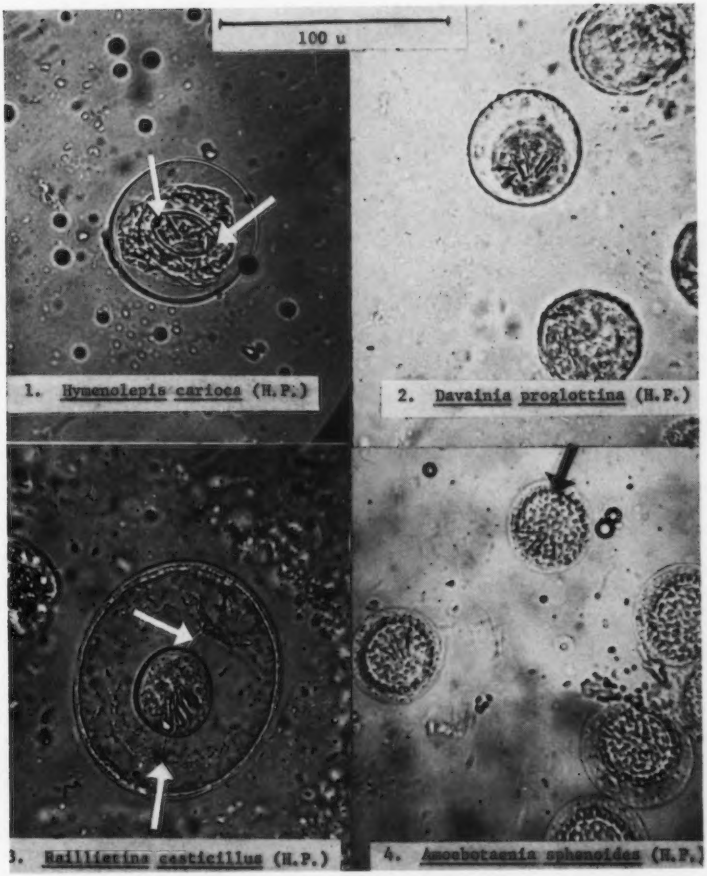

Fig. 1. Hymenolepis sp. distinguished by egg-shaped membrane surrounding the onchosphere and granular accumulations at the poles as indicated by arrows.

Fig. 2. Davainea proglottina has no distinctive structures but may be differentiated by shape and small size.

Fig. 3. Raillietina cesticillus has the largest egg and the funnel-shaped appendages indicated by arrows seen only on mature eggs.

Fig. 4. Amoebotaenia sphenoides has the smallest egg which is characterized by a granular envelope indicated by the arrow.

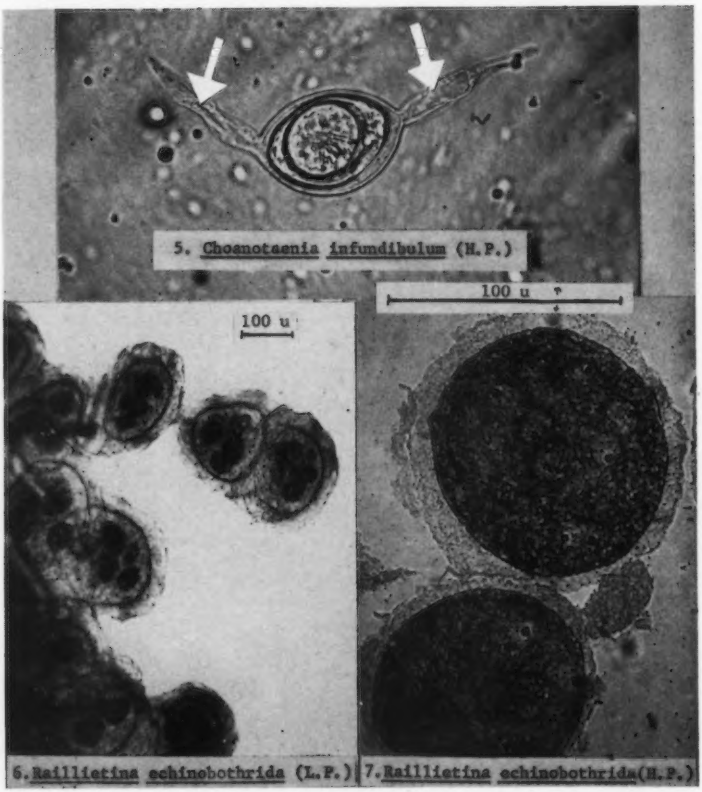

Fig. 5. *C. infundibulum* has very distinctive elongated filaments on mature egg envelopes.

Figs. 6, 7. Encapsulated eggs, 4 to 12 per capsule, are characteristic for *R. echinobothrida*. *R. tetragona* egg capsules are very similar so that species determination must depend on other characteristics. H.P. = high power magnification. L.P. = low power magnification

complete form of one of the larger species. Mascerated, immature proglottids from the larger species contain no distinctive structures. Terminal proglottids of these two small forms contain well-developed onchospheres of rather small size (figures 2 and 4). *A. sphenoides* has a distinctive granular-like layer in a membrane surrounding the six-hooked embryo.

Two of the six larger forms, *Hymenolepis carioca* and *H. cantaniana*, are very narrow, thread-like and easily broken. Several hundred of these worms are frequently found in a single intestine. If this thread-like description fits the specimen, check for the distinctive eggs of the genus *Hymenolepis* (figure 1.) Species identification between these two may be roughly determined by the great difference in strobilar length. Scolex structures differ in these two species but due to the fragility of these specimens, scoleces may be difficult to recover.

The four, large, robust species of tapeworm require preparations of gravid proglottids for egg study. *Raillietina tetragona* and *R. echinobothrida* may readily be distinguished from the other two species since the eggs are contained in multiple capsules. There is no practical means of distinguishing between the egg capsules of the two species. Sawada[4] lists 6 to 12 as the number of eggs in an egg capsule for *R. tetragona* but 3 to 8 for *R. echinobothrida*. Ransom[3] gives the number as 6 to 12 for both species. This character does not appear to be reliable enough for species identification in itself. It is thus necessary to include the size and location of the cirrus pouch and the genital pore, or distinctive scolex characters may be used. Genital pore characteristics may be readily recognized in living material by comparison of water mounts with figures 8 and 9. Since ants are the only reported intermediate hosts of these two species, species identification is unnecessary to suggest control measures. However, *R. echinobothrida* causes nodular pathology and species identification is desirable for flock prognosis.

Raillietina cesticillus and *Choanotaenia infundibulum* have single eggs with distinctive envelopes. *R. cesticillus* is large with funnel-shaped filaments between some of the inner membranes (figure 3). *Choanotaenia infundibulum* has two distinctive elongated filaments attached to the outer membrane (figure 5). These filament characteristics are seen only in entirely mature onchospheres.

INTERMEDIATE HOST CONTROL

Although knowledge of possible intermediate hosts is incomplete with some species, enough information is now

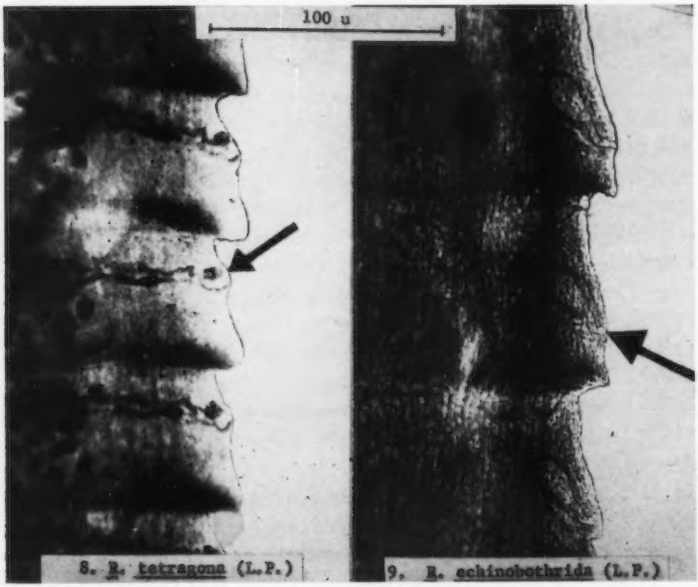

Fig. 8. Raillietina tetragona may be distinguished by the location of the genital pore (arrow) in the anterior half of the proglottid as compared to its location in the posterior half in *R. echinbothrida.*

Fig. 9 R. echinobothrida with genital pore in posterior half of proglotted. Note large cirrus sac as compared with sac in *R. tetragona* L.P. = low power magification. H.P. = high power magnification.

available that it may be used in making management recommendations in poultry practice. Thus far, almost no use has been made of the extensive research information for intermediate host control. Table 1 lists the generally recognized intermediate hosts, and compares the pathogenicity of tapeworms by species.

Slugs and snails. Slugs and snails are commonly controlled in gardens through the use of metaldehyde baits available in most seed stores. Directions of the manufacturer should be followed in tapeworm control programs taking care to exclude birds from access to the poison.

Ants. Jordan[1] gives the following directions which may be used for ant control: "The most complete and long-lasting control will result from a broadcast application of chlordane or dieldrin. The granular form of these insecticides is the handiest to use. Broadcast one pound of 10% chlordane granules or one pound of 5% dieldrin granules per 1,000 square feet. The granules can be broadcast by hand if rubber gloves are used or they can be applied with a lawn seeder." Water the insecticide into the soil before permitting birds into the area.

Beetles. Grain beetles (*Tribolium sp.* or *Tenebrio sp.*) commonly act as intermediate hosts for three species of tapeworms (*R. cesticillus, C. infundibulum* and *H. carioca*). These insects multiply around the feed room and feeders. A thorough clean-up of old feed sacks, unused stored feed and the moving of feeders when beetle development occurs under them, should be recommended. Feed bins should be emptied before refilling. A residual spray such as four pounds of 50 percent wettable DDT in 10 gallons of water used at the rate of 2 gallons per 1000 square feet of surface will control these beetles.

Ground beetles which are important with *R. cesticillus* and dung beetles with *H. cantaniana* will be reduced in number by a heavy application of general insecticides such as recommended for ants.

House flies. Flies (*Musca domestica*) constitute a major problem with which most poultrymen are familiar. It is certain they are important intermediate hosts for *C. infundibulum,* although grain beetles or grasshoppers also may be involved. Populations of adult flies which are the intermediate host form, may be reduced by poison bait stations using Dipterex, Korlan, or Malathion. Housefly populations are reduced most rapidly by control measures directed against larval breeding areas although this will not reduce the number of infected intermediate hosts. Weekly spraying of manure dropping pits with these same insecticides generally is effective. Any fly control program instituted for tapeworm control by the poultryman will be beneficial in control of other poultry and human disease problems.

Grasshoppers. Although these insects can transmit *C. infundibulum,* it seems unlikely they are of frequent enough importance to suggest specific control programs unless such a program is instituted also to prevent crop damage. Grasshoppers also act as an intermediate host for the most common turkey tapeworm, *Metroliasthes lucida.* Aldrin, chlordane, heptachlor and toxaphene are used in chemical control to protect crops from this insect.

Earthworms. Since *A. sphenoides* is not considered very pathogenic, a control program against this species alone is not indicated. It has been shown, however, that about double the recommended dosage of insecticides used in ant control probably will kill earthworms. This may be considered if other tapeworm species are involved.

ACKNOWLEDGMENTS

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SUMMARY

1. A series of photomicrographs together with a table are presented to assist in rapid identification of the eight common species of chicken tapeworms. Suggested control measures directed against specific intermediate hosts and flock prognosis with a knowledge of severity of pathogenicity can be much more accurate after species identification has been completed.

2. Suggestions for the control of various types of intermediate hosts are presented as an approach to tapeworm eradication programs in poultry flocks.

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STUDIES OF EGG TRANSMISSION OF PPLO IN CHICKENS*‡

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THE widespread occurrence and economic significance of chronic respiratory disease (CRD) in chickens in the United States is, at this time, a well documented fact. It is also accepted by research workers in poultry pathology that pleuropneumonia-like organisms (PPLO) play an important role in

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this disease. Although viral agents, such as Newcastle disease and infectious bronchitis, and bacterial agents such as certain coliform strains, can also play an important role in the disease under field conditions, the evidence available at present indicates that PPLO are essential etiological factors in this disease.

The occurrence of egg transmission of PPLO and the significance of this process in the spread of CRD have also been established. The pertinent literature on this subject has recently been reviewed by Calnek and Levine.[2] Because of the obvious importance of egg transmission in the spread of CRD and the possibility that the disease might be controlled at this stage of its cycle, a series of experiments was carried out to study this process. Since previous field and experimental evidence had indicated that the rate of egg transmission of PPLO might be rather low and irregular under natural conditions, attempts were made to find experimental methods of producing a high and regular egg transmission rate. Such an experimental technique would simplify the study of the process of egg transmission and the evaluation of methods for preventing this mode of spread of CRD.

The experiments described herein were done over a period of four years. Since the data from the California and the New York laboratories bore on the same subject, it was decided to combine the results in a single paper.

MATERIALS AND METHODS

Since these experiments were carried out at different times and different places, the materials and techniques varied. A description of the media and some of the techniques used are given below.

Adler's blood slope overlay medium. This medium consisted of a blood slope prepared from Bacto blood agar base (Difco) containing 10 percent fresh defibrinated horse blood. To this agar slope was added enrichment broth consisting of Bacto PPLO broth (without crystal violet) to which was added 10 percent horse serum, penicillin (1000 units per ml) and thallium acetate (1:2000 concentration). In some of the ex-

periments, this broth was also supplemented with 1 percent yeast hydrolysate (Nutritional Biochemical Co.).

PPLO agar medium. This medium consisted of Bacto PPLO agar (Difco) to which was added 10 percent horse serum and inhibitors as in the previous medium. In some cases 1 percent yeast hydrolysate was also added. In some cases the horse serum was replaced by 1 percent PPLO serum fraction (Difco).

Modified Grumbles' medium. This medium consisted of phenol red broth base (Difco) to which was added carbohydrate (1 percent dextrose or 1 percent lactose), 1 percent PPLO serum fraction and inhibitors as described previously.

Culture of fresh eggs or unfertilized eggs. In general, fresh eggs were cultured by cracking the shell, pouring off the albumen and then removing 0.1 ml of yolk with a sterile pipette and seeding into medium. In some cases the albumen was also cultured in a similar manner.

Culture of fertile eggs. These eggs were incubated and candled at 5 days and daily thereafter. Infertile eggs or dead embryos were cultured after 5 days of incubation. The other embryos were cultured as they died or were removed from the incubator for culture at 19 or 20 days of incubation. In some experiments, a sample of yolk was removed directly from the embryo yolk sac with a pipette. In others, the whole embryo was dropped into a bottle containing 30 ml of broth, shaken thoroughly to suspend the yolk and a sample removed for culture in media.

In some experiments an attempt was made to effect a higher incidence of egg transmission of PPLO by using a modification of the Van Drimmelen[5] method of intraperitoneal insemination of birds. By this technique, the PPLO were introduced directly onto the surface of the ovary. Preliminary injections with dyes indicated that the ovules were literally bathed with organisms. The procedure was done in the following manner: The hen was placed on its right side, with its legs toward the right of the operator. A 4-inch 18 gauge hypodermic needle, introduced at a point 1 cm ventral to the anterior extremity of the pubic bone, was passed into the

abdominal cavity in an antero-medial direction pointing toward the right lung. The needle usually was inserted to a depth of about 8 cm in White Leghorn hens.

Differences in technique from what has been given above will be described in the account of the individual experiments.

RESULTS

Experiment 1-A (New York). The birds in this experiment were 60 Single Comb White Leghorn pullets in full production and came from the closed flock of the poultry disease experimental farm. They had never been vaccinated or exposed to any respiratory disease. The pullets were placed in batteries in an isolated building and allowed a period of acclimation to stabilize egg production. These birds were inoculated into the posterior thoracic air sac with one ml of a chicken embryo culture (VH strain) of PPLO which had been isolated from a field case of CRD in chickens. This strain was selected for use because in a previous screening trial it had produced well defined air sacculitis in adult chickens. Five control birds were tested and found free of neutralizing antibodies for Newcastle disease and infectious bronchitis and on necropsy contained no gross lesions in their respiratory tracts. No PPLO were isolated from their respiratory tracts.

The inoculation produced only a mild respiratory reaction in the birds and little or no effect on egg production. Some of the birds which were removed for necropsy had only mild gross and microscopic lesions typical of CRD. Five hundred fresh, unfertilized eggs laid by these birds were examined starting at the time of inoculation of the hens through a period of about three months. Yolk samples were cultured in modified Grumbles' media with dextrose and lactose. No PPLO were isolated from these yolks.

Experiment 1-B (New York). The source of birds, strain of PPLO, and route of inoculation were the same as those used in experiment 1-A. However, in this case, males were placed in the laying pen with 70 hens to produce fertile eggs. These eggs were identified by trap nesting the hens. They were incubated and pedigree hatched at weekly intervals. At

hatching time, those day old chicks not selected for rearing and all of the culls, pips, and dead embryos were examined for CRD lesions. No lesions were found in over 2500 of these eggs examined over a period of 16 weeks after inoculation of the hens.

Six lots of these chicks (70 to 100 per lot) were kept in isolation units. The six lots of chicks came from eggs laid the 2nd, 3rd, 6th, 8th, 9th, and 10th weeks after inoculation of the hens. Over a 15 week period, samples of from 10 to 30 chicks were removed on two and in some cases three occasions for examination. When the chicks were 8 to 10 weeks of age, they were infected with fully virulent infectious bronchitis virus (Bickhart strain). In every case at least two groups of chicks were sampled following exposure to bronchitis. No gross or microscopic evidence of CRD was found in these chicks before or after infection with infectious bronchitis

Experiment 2-A (New York). Because of the failure to secure evidence of egg transmission in 1-A and 1-B with experimentally infected chickens, experiments 2-A and 2-B were carried out similarly with naturally infected White Leghorn hybrid pullets 6 to 7 months of age. The birds in this experiment came from a commerical flock in which CRD had been diagnosed and PPLO isolated. Symptoms were first noted in this flock about 3 weeks previously. The birds were still in the active stages of the disease at the start of the experiment. They were selected for the experiment on the basis of two criteria: first, the presence of respiratory symptoms and second, evidence of egg production.

A group of 90 of these birds was transported to the laboratory grounds, placed in a laying pen with males and trapnested. The eggs were incubated weekly and chicks examined for CRD lesions at hatching time. Twenty-four hatches averaging 200 chicks each were examined. CRD lesions were found in embryos, dead in shell, in pips, and live chicks from many of the hatches. The percentage of birds or embryos with lesions fluctuated from week to week and followed no particular pattern. These percentages ranged from 0 to 9.5 percent. However, in the later stages of the experiment, the

number of embryos with lesions became so few and intermittent, that further work became impractical.

Six groups of 100 chicks each, derived from some of the 24 hatches were raised in isolation units. The results secured with these chicks are presented in table 1. It is evident that lesions of CRD resulting from egg transmission were found in day old chicks and embryos. In spite of this carry over of PPLO into the chicks, no clinical symptoms occurred in the birds either before or after exposure with various viral and bacterial agents. Furthermore, there was a consistent failure to isolate PPLO from these lesions. Only relatively mild lesions were seen when the birds were examined. In none of the experimental groups did there develop a clinical outbreak of CRD.

Experiment 2-B (*New York*). Eighty hens from the same source as that used in experiment 2-A were placed in laying batteries. Fresh, infertile eggs laid by these birds were cultured for PPLO. Yolk samples were cultured from over 1000 eggs and albumen samples were cultured from over 500 eggs. One hundred eggs were cultured by dropping the whole yolk into a bottle containing 30 ml of broth, shaking to suspend the yolk material and then inoculating an aliquot into media as already described. All cultures in this experiment were carried out in modified Grumbles' carbohydrate media (dextrose and lactose). No PPLO were isolated from these eggs.

Experiment 3 (*New York*). In this experiment, an attempt was made to reproduce the pattern of a severe CRD outbreak by inoculating susceptible laying hens with a mixture of two strains of PPLO and infectious bronchitis virus. (IBV, Bickart strain) into the air sacs. PPLO strain 85 (later shown to be a C type) was in the form of a broth culture, and the second PPLO strain, Adler, (later shown to be an S_6 type) was in turkey sinus exudate .

The hens, which were of four age groups (1 to 4, table 2) came from the poultry disease experimental farm and were free of all respiratory diseases. The birds were wing-banded and placed together in a laying pen with sufficient males. Four additional groups (5 to 8, table 2) were inoculated with vari-

TABLE I

TABLE I

Results of examinations and exposure of groups of 100 chicks, reared in isolation and derived from a flock naturally infected with CRD. (experiment 2-A)

Group	Number of weeks after onset of CRD in hens	% lesions in embryos examined	Pre-exposure examination of chicks	Age of chicks in weeks when exposed	Mode of exposure	Examination of chicks four weeks post-exposure
1	13	2	AM* 10 neg. PM 10 neg. culture	10	Sprayed with inf. bronchitis virus Crawley virus	AM 20 neg. PM 4 ++ † 7 + 9 neg. culture - 20 neg.
2	14	1	AM 10 neg. PM 10 neg. culture	9	Same as group 1	AM 20 neg. PM 9 + 11 neg. culture - 20 neg.
3	17	2	not done	6	Infected with inf. bronchitis virus	AM 20 neg. PM 7 + 13 neg. culture - 20 neg.
4	18	1	not done	5	Same as group 3	AM 20 neg. PM 6 + 14 neg. culture - 20 neg.
5	19	3	AM 10 neg. PM 1 ++ 1 + 8 neg. culture - 10 neg.	15	Sprayed with inf. bronchitis virus, B1 Newcastle & *Escherichia coli* culture	AM 15 neg. PM 1 ++ 8 + 6 neg. culture - 15 neg.
6	21	5	AM 10 neg. PM 3 ++ 4 + 3 neg. culture - 10 neg.	13	Same as group 5	AM 15 neg. PM 1 ++ 8 + 6 neg. culture - 15 neg.

*AM=ante mortem
PM=post mortem

† + = clouding or thickening of air sacs
++ = same as † and in addition with watery or mucoid exudate
Numbers refer to number of chicks in each category

ous combinations of agents. These birds were combined later with the birds of groups 1 to 4 in the laying pen. Tracheal swab cultures and plate agglutination tests for PPLO conducted prior to inoculation were negative. After inoculation, tracheal swab cultures and plate agglutination tests were done every 2 weeks on a sample of birds from each group.

Subsequent to inoculation, the birds were trapnested, eggs identified and all eggs cultured. The eggs were incubated and candled at daily intervals, starting on the fifth day of incubation. All infertile eggs or dead embryos were cultured on the fifth day. Other dead embryos that were detected on subsequent daily candling were also cultured. All embryos still living at 14 days of age were cultured by dropping the whole embryo together with its membranes in broth, shaking and inoculating a sample of the suspension into modified Grumbles' media (dextrose and lactose).

The design of this experiment and the effect of the inoculation on the birds are summarized in table 2. All of the birds developed respiratory symptoms by the second day after inoculation. Most of the birds continued to have respiratory symptoms 2 and some 3 weeks or more after inoculation. Symptoms which persisted for periods up to several months were typical of those seen in a natural outbreak of CRD. Those birds which were in egg production at the time of inoculation stopped laying by the end of one week. Egg production was not resumed until 35 days after inoculation.

It can be seen in table 2 that this combined inoculation with IBV and PPLO also produced a marked mortality which varied from group to group. Birds in heaviest production were much more severely affected than those in light production or those not yet in production. Mortality began 4 days after inoculation and continued during a period of 6 to 7 weeks. Peritonitis and nephritis were found in birds that died early. A few birds had pericarditis, perihepatitis or both. In the birds that died later in the course of the disease, air sac infection and sinusitis were found. Later in the experiment, some birds with only mild respiratory symptoms were ob-

TABLE II

Results of inoculations of breeder hens with mixture of infectious bronchitis virus (IBV), PPLO strain 85, PPLO strain Adler and with each agent singly. (experiment 3)

Group	Number of birds	Age in months	Level of egg production at start	Inoculum	Mortality	PPLO isolation from trachea after two months	PPLO plate agglutination test after two months (3)	No. birds laying infected eggs Total no. birds from which eggs were cultured	No. infected eggs/ Total eggs cultured from all surviving hens in group
1	31	8	83%	IBV, PPLO 85, Adler	19	7/7+*	4/4+*	2/8	2/65
2	30	4½	0	IBV, PPLO 85, Adler	3	8/8+	4/4+	4/24	11/270
3	15	5	0	IBV, PPLO 85, Adler	4	4/4+	2/2+	2/9	2/83
4	9	5½	10	IBV, PPLO 85, Adler	5	2/2+	2/2+	1/1	1/15
5(1)	12	5½	10	IBV (2)	1	2/2+	2/2+	2/6	2/67
6(1)	10	9	?	IBV (2)	0	2/2+	2/2+	0/6	0/72
7(1)	10	9	?	IBV (2) PPLO 85	2	2/2+	1/2+	0/4	0/22
8(1)	10	9	?	IBV (2) PPLO Adler	3	2/2+	2/2+	0/3	0/8

(1) Groups 5, 6, 7, 8 combined with groups 1-4 in a single pen one month after groups 1-4 were inoculated.
(2) Groups 5, 6, 7, 8 infected in separate pens 11 days after 1-4.
(3) Lederle PPLO antigen used.
* Number successful cultures/number birds cultured.

served in a semi-comatose condition for several days before they died. On necropsy they were found to be emaciated and affected with a severe nephritis. PPLO were reisolated from almost all of the birds that died and in addition, various species of bacteria including *Pseudomonas spp.*, gram positive and gram negative rods, and cocci.

In tables 2 and 3 are recorded the results of the egg examinations for PPLO which were continued for 40 days after the birds resumed production (the 75th day after inoculation). Only two of the 11 birds that produced infected eggs laid more than one (table 3). In these birds, the interval between non-infected and infected eggs varied very widely. Bird R62 laid an infected egg at the start of production after infection but failed to produce another among the following 23 eggs produced in 28 days. On the other hand, R55 laid 15 negative eggs during the first 20 days before producing six infected ones of the nine laid during the following 15 days. Although the number of birds producing infected eggs at one time or another was fairly high, (18 percent), the number of infected eggs produced by the birds was quite low, (3 percent).

It is also interesting to note that the percentage of embryos that remained alive up to 14 days of incubation appeared to depend on the intensity of egg production at the time of infection. Prior to infection, the percentage of live embryos at 14 days produced by the flock from which the experimental birds were drawn was 71 percent. Group 1, which was up to 83 percent production before infection, produced only 3 percent live embryos at 14 days from the eggs laid subsequent to infection. Groups 2 and 3 (in the same pen and served by the same males), which were not yet in production at the time of infection, produced 43 and 39 percent live embryos, respectively.

At the end of this 40 day period of egg culturing (75th day after infection), a series of other experiments was carried out with the same birds. For 6 weeks (76th to 117th day after infection), all eggs were incubated and hatched. The hatched chicks, pipped embryos and dead in shell embryos were ex-

TABLE III

Sequence of egg production of "shedder" hens following infection with strain 85 and Adler strains PPLO and infectious bronchitis virus. The numbers refer to day after infection on which an egg was laid. The italicized numbers indicate that the egg yielded PPLO on culture. (experiment 3)

Group	Bird no	Days after infection when eggs were laid.
1	R 9	35, 36, *38*,* 41, 42, 43, 44,
1	R 27	35, *49*,
2	R 35	*35*, *38*, 39, *42*, 43, 44, 45
2	R 49	35, 37, 40, 41, 43, 44, 45, 51, 53, 54, *56*, 57
2	R 55	35, 40, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, *55*, 57, 58, 59, *60*, *61*, *64*, *66*, *69*
2	R 56	35, 36, 45, 46, 47, 48, *52*
3	R 62	*35*, 36, 37, 38, 40, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 69, 62
3	R 72	35, *38*
4	R 79	35, 36, 47, 49, 50, 52, 54, 57, 59, 62, 68, 70 *72*, 74
5	Y 37	35, 37, 38, 39, 50, 52, *56*
5	Y 51	35, 36, 37, 39, 40, 41, 42, 44, 46, 47, 49, *51*, 52

* Italicized figure represents infected egg.

amined for lesions. In addition, all live chicks and pips were cultured for PPLO. The results of this phase of the experiment were as follows:

586 infertile or dead by 18th day.
168 dead on 21st day.
50 pipped. Six of these had respiratory tract lesions from which 2 PPLO isolations were made.
217 hatched. Eight chicks had respiratory tract lesions from which 5 PPLO isolations were made.

Of the 11 hens that had been found to shed organisms in their eggs previously, only six hens passed detectable infection to their progeny. No new "shedder" hens were uncovered. Tracheal swabs from a sampling of both "shedders" and "non-shedders" yielded PPLO in every case.

No examinations were made for the next 21 days. Since "shedder" hens had been identified by cultural examination of their eggs and chicks, an attempt was made to rear chicks from these hens and also from the "non- shedder" hens. Eggs were collected from these two groups of hens for a one week period (140 to 146 days after inoculation) and again for a 2 week period (203 to 217 days after inoculation). The "shedder" hens produced only one viable chick in the first period and none in the second. The "non-shedder" hens produced 24 and 21 chicks respectively in the two periods. None of these chicks were found to be infected with PPLO even after being raised in isolation for 6 weeks and then stressed with infectious bronchitis virus. Necropsy, culture and plate agglutination tests were all negative four weeks after bronchitis infection.

Two more series of cultures of eggs were made. All eggs laid during the periods from 196 to 202 and 218 to 245 days after infection were cultured. This included 64 eggs laid by seven "shedder" hens. These eggs yielded no PPLO. However, two PPLO isolations were made from two eggs out of 713 examined laid by 54 hens previously listed as "non-shedders". These eggs came from two birds (one each) which previously had produced clean eggs and chicks.

At the end of the experiment, ten hens from each group

("shedders" and "non-shedders") were necropsied and cultured. None of these birds had respiratory symptoms at this time. PPLO were isolated from the trachea in seven of nine "shedders" and eight of nine "non-shedders". On the other hand, neither group of birds yielded PPLO on culture of the ovarian tissue.

The post mortem findings in the "shedder" group seemed to indicate that the infection in these birds was more active than that in the "non-shedders". Nine of ten "shedders" had caseous exudate in the abdominal cavity while only one of ten "non-shedders" had peritoneal or air sac exudate. Fibrous adhesions were seen in both groups.

Experiment 4 (*California*). In this experiment, a group of 17 20-month-old Single Comb White Leghorn hens which had CRD during their growing period were used. At the time of inoculation, these birds were free of symptoms and negative to the serum-plate agglutination test. A series of control eggs was collected during the week prior to inoculation. Seventeen of these hens were injected by the modified Van Drimmelen technique with one ml of a culture of the S_6 strain of PPLO (48th media passage). These birds were housed in a pen with two males and their eggs collected and incubated. Infertile or dead embryos were cultured after 5 days of incubation. The other embryos were cultured as they died or were killed for culture on the 18th or 19th day of incubation.

The eggs were cultured by removing 0.1 ml of yolk or allantoic fluid with a sterile pipette and seeding this material into broth. The embryos themselves were cultured after being washed in sterile saline and minced with a sterile scissors. About 0.1 ml of minced embryo was used for the inoculum. Where possible, all three of these materials, yolk, allantoic fluid, and embryo, were cultured from the same egg.

The culture technique consisted of seeding the inocula into Adler's blood slope overlay medium. Each culture was carried through three serial passages on overlay broth before being considered negative. Each overlay broth was subcultured to a PPLO agar plate.

The inoculation did not appear to cause any symptoms in the birds. In this experiment, the birds were not trapnested but handled as a group. The results of egg culture are summarized in tables 4 and 5. No PPLO were found in the control eggs. In these tables, it may be seen that during the course of the experiment approximately 9 percent of the eggs contained PPLO and that positive eggs were detected for a period of at least 90 days. It is also evident that the yolk yielded more isolations than the other tissues tested and that the majority of isolations were made from embryos dying between the 6th and 14th day of incubation.

Experiment 5 (*California*). The procedure and materials in this experiment were identical to those in experiment 4 except that the birds were trapnested and records kept on the individual birds and that only yolks were cultured. There were 20 hens and 3 roosters in this group. Eight of the 20 hens were in production at the time of inoculation. Of the eight, four birds laid infected eggs. None of the other birds which came into production more than 38 days after infection shed any detectable organisms in their eggs. The individual laying patterns of the "shedder" hens are presented in table 6.

Experiment 6 (*California*). The general procedure used in the two preceding experiments (4 and 5) was also used in this experiment. The birds in this experiment were 8-month-old Single Comb White Leghorn pullets that were clinically free of disease but were positive to the PPLO serum plate agglutination test. This group of 25 pullets was inoculated with a 10th culture passage of the S_6 strain of PPLO.

The first 138 eggs laid by these birds did not have any unusual embryo mortality during incubation. No PPLO were isolated from these eggs. Later examination of an additional and larger group of eggs from the same hens gave similar results.

Experiments 7-A and 7-B (*California*). Two more experiments were done using the same procedures and PPLO cultures described in experiment 4. Twenty pullets, White Leghorns, used in the first experiment (7-A) came from the

TABLE IV

Recovery of PPLO from embryos dead between the 6th and 14th day. The number of eggs laid in each 10 day period following intraperitoneal inoculation of 17 hens are indicated. (experiment 4)

Time in days	1-10	11-20	21-30	31-40	41-50	51-60	61-70	71-80	81-90	91-100	101-110	111-120
PPLO infected eggs	13	1	5	1	5	1	2	4	3	0	0	0
Total eggs laid	38	26	36	32	32	34	34	38	39	43	40	36

flock of the Poultry Husbandry Department of the University of California at Berkeley. This was an isolated flock which had been free of respiratory disease for several years. These birds were serologically negative and symptom free. Twenty birds used in the second experiment (7-B) came from the same source as the birds in experiment 4. These birds were symptom free but serologically positive.

TABLE V

Distribution of PPLO in chicken embryos from 17 hens exposed intraperitoneally (experiment 4)

Time of incubation	Total eggs	PPLO isolations			Total positive
		Yolk	Allantoic fluid	Embryo	
Dead or infertile at five days	70	6	0	0	6
Dead 6-14 days	358	31	11	16	35
Dead 15-19 days or alive at 19 days	22	0	0	0	0
TOTAL	450	37	11	16	41

PPLO were not isolated from 156 eggs or embryos in either of these two experiments. In both cases the experiment was abandoned when no PPLO were recovered from the eggs laid during the first 3 weeks after inoculation.

Experiment 8 (*New York*). A group of 71 PPLO-free White Leghorn hens from the poultry disease experimental farm was placed in laying cages. The birds were permitted to accustom themselves to the surroundings. No PPLO were isolated from eggs laid by these birds during a period of one week prior to inoculation. The birds were injected by the modified Van Drimmelen technique with a mixture of Adler strain and 293 strain of PPLO (both S_6 type strains). In addition, each bird was given 0.5 ml of culture intratracheally. These hens went out of production 7 to 12 days following in-

oculation. Twelve birds that died were for the most part emaciated, dehydrated, and had marked lesions in their air sacs. About 2½ months after inoculation, the survivors completely recovered and came back into egg production.

TABLE VI

Sequence of egg production of "shedder" hens following intraperitoneal inoculation of S^6 strain PPLO. The numbers refer to day after infection on which an egg was laid. The italicized numbers indicate that the eggs yielded PPLO on culture.

Bird number	Days after infection when eggs were laid.
237 C	6, 12, *18,** *19*, 20, 21, 23, *24*, 27, *28*, *32*, 34, 36, 36, 41, 53, 55, 56, 57
490 D	7, 17, 19, 21, 22, 23, 25, *29*, 31, *33*, *34*, *34*, *41*, 47, 48, *50*, 51, 53, *57*
463 E	*11*, 14, *16*, 17, 19, 20, 21, 23, 51, 53, 55, 59, 62, 62, 64, 65, 67, 68, 70, 71
460 F	21, 21, 48, 49, 50, 52, *54*, 64, 65, 67, 69, 70, 76, 80, 81, 82, 84

* Italicized figure represents infected egg.

Fresh eggs (non-fertile) from these hens were cultured for PPLO by dropping the whole yolk in broth, shaking and subculturing to modified Grumbles' media (dextrose and lactose). Thirty fertile eggs which were secured by artificial insemination of these hens were incubated and cultured as described in experiment 5. No PPLO isolations were made from any of a total of 305 post-inoculation eggs that were cultured.

Experiment 9 (*New York*). Eighty-four PPLO free Single Comb White Leghorn hens taken from a breeding pen of the poultry disease experimental farm were placed in laying cages. A period of three days was allowed for acclimation of the birds and collection of control eggs. The birds were then inoculated into the posterior thoracic air sac with 0.5 ml of a yolk culture of PPLO strain 293 (S_6 type). In addition, each bird was given 0.5 ml of the same culture intratracheally.

The inoculation produced a severe mortality in the hens; 27 of the 84 birds died between the 7th and 98th day after inoculation. Birds dying early in the course of the disease yielded PPLO on culture. Those dying later were negative. Almost all of the birds had marked to severe respiratory tract lesions at post mortem. At least eleven of these birds had nervous symptoms for a period before their death. These consisted either of twitching of the head or of sitting in a semi-comatose manner for a period of several days. Such birds, when necropsied, were severely emaciated and often had a severe nephritis. These findings obviously resulted from the inability of the birds to drink or eat during this period before death.

Three lots of eggs from these birds were cultured for PPLO. The first lot of 130 fertile pre-inoculation eggs and the third lot of 246 eggs, laid from the 97th to 111th days post-inoculation after the birds had been artificially inseminated, were incubated and cultured as described in experiment 3. No PPLO were recovered. The second lot of 395 eggs, laid from the 1st to the 73rd day post-inoculation, were cultured as fresh eggs as described in experiment 8. Three of these were positive. Three birds laid the infected eggs—one on the 13th day post-inoculation (9th egg of 35), a second laid one on the 4th day post-inoculation (1 of 3), and the third hen laid one on the 3rd day post-inoculation (1 of 4).

Experiment 10-A (New York). The next experiment, 10-A, was designed with the purpose of gaining some information about the mechanism of egg transmission. The birds in this trial came from the PPLO free flock of the poultry disease experimental farm. Control eggs were collected for one week prior to inoculation. The inoculum in this trial consisited of a mixture of media and yolk cultivated PPLO strain 293 (S_6 type) containing at least 10^8 organisms per ml.

Fifty Single Comb White Leghorn hens, 14 months of age, were inoculated into the posterior thoracic air sac. The hens, which were kept in a laying pen with males, were trapnested and eggs marked and cultured individually for PPLO after incubation. The technique was similar to that used in

experiment 3 except that the embryos were allowed to reach 18 days of age and a more complex culturing technique was used. This consisted of seeding embryo suspension into Adler's overlay broth and making three serial passages at 3 day intervals. Each overlay passage was subcultured to a PPLO agar plate or a modified Grumbles' maltose or both.

At weekly intervals through the course of the experiment, from the 3rd to the 10th week after infection, hens were removed for necropsy and culture. An attempt was made to necropsy and culture the "shedder" hens as soon as posisble after their eggs were found infected.

At the time of necropsy of each bird, a separate culture for PPLO was made from the trachea, lung, air sac, cloaca, oviduct, and each ovarian follicle over 1 cm in diameter. All PPLO isolated from these tissues or from the eggs were checked to make sure they were the same type (S_6) as the culture with which the birds were inoculated.

The hens went out of production for a period of about 7 to 10 days starting 5 days after inoculation. Fourteen of the hens laid one or more PPLO infected eggs during the ten week period of the experiment. A total of 24 positive isolations was made from 518 eggs cultured. Five birds were repeaters and laid three infected eggs each. PPLO were isolated from the tissues of eight of these 14 "shedder" hens. These isolations were made from the trachea, air sacs, lungs, and cloaca but not from the ovaries (294 individual ova cultured) or oviducts.

Isolations were also made from similar tissues in 9 of the 36 hens in which egg transmission was not detected. There were no significant differences in the severity of air sac lesions observed at necropsy between the "shedders" and the "non-shedders". Birds with marked, moderate, and no lesions were found in both groups. There were no gross pathologic changes observed in the ovaries of either group.

Experiment 10-B (New York). Each of 30 hens, 14 months of age, (from the same source as experiment 10-A) was given four intravenous inoculations with 0.5 ml of PPLO

strain 293 (containing at least 10^8 organisms per ml) during the course of an eight day period. The repeat doses were administered 2, 5, and 7 days after the first dose. Eggs were collected starting at the time of inoculation until the end of the experiment 7 weeks later. These eggs were cultured as fresh eggs. The whole yolks were dropped into broth diluent, shaken and a sample removed for culture. The culture technique was the same as that used in experiment 10-A. Similarly, groups of birds were removed for necropsy and culture 2, 3, 4, 5, and 7 weeks after the first inoculation. These birds were handled as in experiment 10-A.

No PPLO were recovered from any of the 344 eggs laid by these birds. Only two of the hens had significant lesions in the air sacs and lungs at autopsy. Seven of the 30 hens yielded PPLO on culture. These isolations were made from air sacs, lungs, and cloaca as in the previous experiment. However, in this experiment, two of the 10 birds necropsied 2 weeks after first inoculation (one week after the last inoculation) each contained PPLO in one of their ova. No others of a total of 63 ova cultured at this time period from this group of ten birds or the 173 ova cultured during the entire period of this experiment yielded PPLO. None of the 17 eggs laid by the two positive birds yielded PPLO. No cultures were isolated from the oviduct of any of the birds in this experiment.

DISCUSSION

It seems obvious that, so far, none of the experimental techniques employed have given results that are sufficiently reproducible to serve as standard experimental procedures. The introduction of large numbers of PPLO into the air sacs or directly over the surface of the ovary did not always induce detectable PPLO transmission to eggs by laying hens. In repeated experiments with almost identical techniques and cultures, some trials were successful and others failed. Within groups of birds on the same experiment, there were wide variations in the degree of success of egg transmission. A small number of hens would shed organisms repeatedly; a larger group of hens would only lay one or a few PPLO infected

eggs and many similarly treated birds would lay no infected eggs. Even among the individual "shedder" hens, there was little or no consistency to the pattern with which infected eggs were laid.

Because of the incomplete knowledge of the pathogenic and cultural characteristics of avian PPLO, it is difficult to assess the accuracy of our experimental methods. The possibility exists that the inconsistencies found in these results may be due, in part, to shortcomings in our cultural methods for PPLO. This possibility is indicated in the results reported by Fabricant[4] on the comparative efficacy of various cultural techniques for the isolation of avian PPLO. These results indicatd that there is a significant error inherent in the use of all of our present media procedures for the isloation of PPLO.

Another factor to be considered in the evaluation of these and previous experiments on egg transmission is the occurrence of various PPLO types as reported by Adler *et al.*[1] In this series of trials, however, all of the experiments from numbers 4 to 10 were carried out with known pathogenic types (S_6). It was shown by Adler *et al.*[1] that PPLO types which were weakly pathogenic or non-pathogenic for birds (C and N) could also be egg transmitted and produce lesions in embryos and day-old chicks. The significance of these findings still is not clearly understood.

The evaluation of the success of egg transmission by attempting the reproduction of air sac infection in chicks raised in isolation was also made difficult by our inability to reproduce a disease in these chicks as serious as that seen under field conditions. Even when these chicks were infected with various combinations of viruses and bacteria believed to be associated with the field disease, only a relatively mild infection was observed.

Although these previously noted problems complicate the evaluation of the data secured even in the successful transmission experiments, field and experimental evidence seem to point to the intermittent nature of PPLO egg transmission.

Both field and experimental evidence also seem to indicate that the rate at which egg transmission occurs decreases as the acute stage of the disease wanes and the disease becomes more chronic. It seems evident that the rate of egg transmission eventually decreases to a very low level or even ceases entirely. This is indicated by the fact that clean progeny have been raised from known infected hens Unfortunately, the usefulness of this information is still limited by our inability to predict the pattern of egg transmission in any given flock even under experimental conditions.

The evidence presented in experiment 10 would seem to indicate that egg transmission of PPLO is not due to a true reproductive tract infection, such as is seen in the ovary and oviduct of pullorum disease. Only two PPLO isolations were made from 173 ovarian follicles cultured following repeated intravenous inoculation of the hens with large numbers of organisms. These two isolations were made one week after the last intravenous dose. Sixty-one other isolation attempts made at the same time were negative. On the other hand, no PPLO isolations were made from any of 294 ovarian follicles cultured after inoculation into the air sacs. No PPLO were isolated from the oviduct in hens inoculated by either route. These findings, together with the distribution of lesions and isolations in experimentally and naturally infected hens, lead us to suggest that egg infection is due to the contamination of the ovules at the time they leave the follicle or in the infundibular portion of the oviduct. The possibility that this contamination occurs is made likely by the contiguity of the abdominal air sacs to the ovarian follicles and to the thin-walled infundibular region of the oviduct. The data presented in table 5 also indicate the significance of the yolk as the probable route of embryonic infection as well as the tissue of choice from which to make isolations.

Although the wide variety of reactions observed in the hens following inoculation make it difficult to generalize, several specific items of interest were noted. One was the relationship between intensity of egg production and the reaction to a combined infection with PPLO and infectious bron-

chitis virus (experiment 3 and table 2). Both the mortality and the effect on embryonic viability were much more severe in birds in full egg production than in those at a low rate of production or just ready to start production. However, it is difficult to say how much of these deleterious effects were due to the bronchitis virus alone since adequate controls were not available.

Another interesting clinical point was the occurrence of nervous symptoms in two of the groups of hens inoculated with PPLO (experiments 3 and 9). The nature and cause of these nervous symptoms are probably explained by the later findings of gross and microscopic lesions of PPLO encephalitis in an adult chicken inoculated with PPLO strain 293 in an experiment not described in this paper. This was the same strain of PPLO used in experiment 9. The lesions were essentially similar to those described in the turkey by Cordy and Adler.[3]

ACKNOWLEDGMENTS

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SUMMARY

The experiments, carried out over a period of four years with 774 hens, were done to assess the conditions under which hens lay eggs with PPLO infection. Particular attention was given to the frequency and sequence of appearance of PPLO infected eggs over a period of time, the percentage of infected eggs produced, and the fate of the progeny. Hens infected naturally and experimentally with PPLO were used. Some eggs produced by the hens were cultured fresh but most were incubated and the embryos cultured for PPLO. In a number of experiments, chicks were hatched from the eggs, reared in isolation, and then infected with other respiratory diseases in efforts to induce chronic respiratory disease.

1. Air sac inoculation of 60 hens with a PPLO culture

failed to effect transmission of PPLO in 500 non-fertile eggs examined. Similar negative results were obtained when 1000 eggs were examined that were produced by 80 naturally infected hens.

2. Seventy hens producing fertile eggs were inoculated with a PPLO culture into the air sacs. No gross lesions were found in 2500 day old chicks, pips, and culls. Six lots of chicks (70 to 100 per lot) were reared in isolation and exposed to virulent infectious bronchitis virus at 8 to 10 weeks. No evidence of CRD could be demonstrated in these chicks.

3. Ninety naturally infected hens produced about 4800 embryos, dead in shell, pips, and live chicks over a 24 week period. Gross CRD lesions in the respiratory tract were seen in 0 to 9.5 percent of the specimens. There was much fluctuation from week to week with no obvious pattern. Toward the end, the number of lesions were very few. When 600 chicks (6 hatches) from the same hens were reared in isolation and exposed to various viral agents and bacteria at ages from 5 to 13 weeks, no severe outbreaks of CRD were induced.

4. Mixtures of bronchitis virus and two strains of PPLO (S_6 and C types), bronchitis and S_6 PPLO, bronchitis and C type PPLO, and bronchitis alone were used as inoculums in eight groups of birds (experiment 3). There were 127 hens in all from 4½ to 9 months of age. Mortality was severe (60 percent) especially in the group in heaviest production. Symptoms and lesions of CRD were produced. During the 75 days following infection, eleven out of 63 birds laid eggs infected with PPLO. Only two of the eleven produced more than one infected egg. There was no consistency or pattern in the time of appearance of infected eggs. In some instances, many clean eggs preceded the infected one. In one case the first egg examined was infected and was followed by 24 non-infected ones.

Of the 11 "shedder" hens, six produced progeny with gross CRD lesions (8 out of 217 chicks hatched from all the birds). Between the 145th and 245th day after infection none of the 6 surviving "shedder" hens produced infected eggs.

Two of the 54 hens previously listed as "non-shedders" laid one infected egg each. At the termination of the experiment, PPLO could be isolated from the tracheas of "shedders" and "non-shedders" but not from the ovaries

5. Six trials were done with 173 laying hens wherein PPLO cultures were injected intraperitoneally and the inoculum deposited in the immediate vicinity of the ovary. Four of the attempts failed to result in egg infection with PPLO. Four hens out of 17 in one trial yielded 9 percent infected eggs out of a total of 450. The greatest success in isolating PPLO was from the yolk, embryo, and allanto-amnionic fluid in descending order. In the second successful trial, four out of eight birds passed infection to the eggs. Three of these birds were multiple "shedders." The time of appearance of the infected eggs could not be predicted.

6. In the 9th experiment, 27 out of 84 laying hens inoculated with PPLO strain 293 (S_6 type) died with severe respiratory tract lesions. Eleven had nervous symptoms before death. Cultures of 395 eggs laid between the 1st and 73rd day were made. Three birds laid one infected egg each between the 3rd and 14th day after infection. None of the 246 eggs laid from the 97th to 111th days post infection yielded PPLO.

7. Fourteen of the 50 hens inoculated with PPLO strain 293 (S_6 type) laid 24 infected eggs out of 513 laid during 10 weeks. PPLO were isolated from trachea, air sacs, lungs, and cloaca of eight of the "shedders." However, no PPLO were isolated from the oviducts or 294 individual ova that were cultured. Nine of the 36 "non-shedders" yielded similar results with cultures of their tissues. There were no significant differences between the gross lesions of "shedders" and "non-shedders." The ovaries appeared normal, grossly.

8. When 30 hens were given four intravenous injections of PPLO strain 293, no evidence of transmission to the egg was found after 344 eggs were cultured during 7 weeks. Tissues from seven of the 30 hens yielded PPLO on culture. Two birds of 10 necropsied one week after the last inoculation yielded PPLO from their ovaries. However, none of the 17

eggs laid by these birds was infected. None of the other 171 ova cultured were infected.

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2. Georgia	2.61	2.58	2.13	2.12
3. Alabama (Test #1)	2.21	2.21	2.41	2.54
4. Pennsylvania (Field Trial #3)	2.88	2.89	2.55	2.54
5. Pennsylvania (Field Trial #4)	3.20	3.20	2.48	2.48
6. North Carolina	3.19	3.18	2.30	2.31
7. Mississippi	2.25	2.23	2.51	2.50
8. New York (9 weeks)	3.49	3.40	2.40	2.40
9. Rhode Island	2.49	2.34	2.34	2.40
10. Alabama (Test #2)	2.60	2.53	2.00	2.04
11. Connecticut	3.07	3.05	—	—
12. Pennsylvania (Field Trial #5—9 weeks)	3.56	3.59	2.50	2.51
13. Maine	3.36	3.31	2.17	2.23
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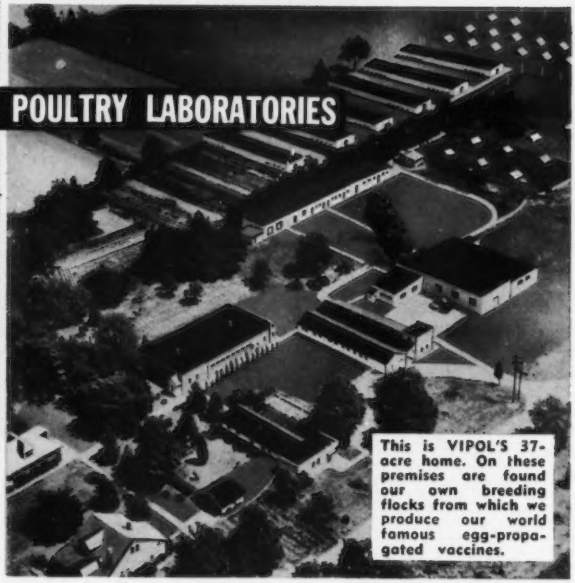

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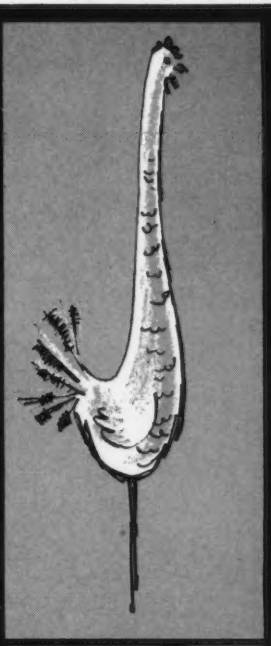

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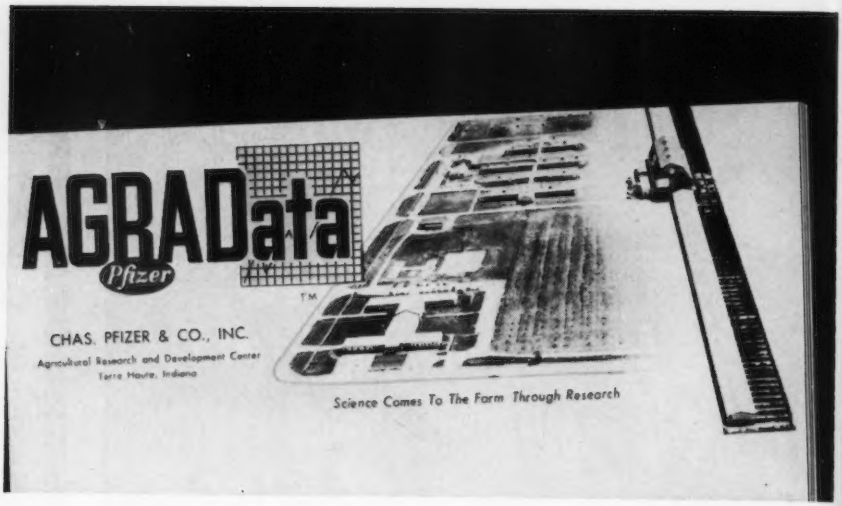

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